Amputation and Heat Induced Protein Synthesis in the Regenerating Forelimb of *Notophthalmus viridescens*

by

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Abstract

This thesis compares the responses of regenerating forelimb tissues of the newt *Notophthalmus viridescens* to the stresses of hyperthermia and mechanical injury of amputation. In particular, both quantitative and qualitative changes in the synthesis of soluble proteins in stump tissues, including those of the heat shock protein family (HSP70-like) were examined.

Results from SDS-PAGE fluorography indicate that the trauma of amputation mimics the heat shock response both quantitatively and temporally in its transient repression of the synthesis of most normal cellular proteins, and qualitatively, in the localized expression of two unique proteins (hsp30 and hsp70). Fluorography of proteins separated by two-dimensional gels revealed that the localized, amputation induced 70kDa protein (amp70) was distinct from the more basic newt hsp/hsc70 isoforms. Although limb amputation resulted in an increase in the synthesis of HSP70 mRNA analogous to that induced by heat shock, amp70 did not cross-react with murine monoclonal antibodies directed against both the inducible and cognate HSP70 proteins of the human. Thus, the possible relationship of amp70 to other members of the HSP70-like protein family remains unclear.

Western analyses indicated that the levels of the constitutive form of HSP70 (hsc70) were found to be regulated in a stage-dependent manner in the distal stump tissues of the regenerating forelimb of the newt. The highest levels were found in the mid-late bud stage, a period during which rapidly dividing blastema cells begin to redifferentiate in a proximodistal direction. Immediately after amputation, hsc70 synthesis and accumulation was depressed below steady-state levels measured in the unamputated limb. The results are discussed in light of a possible role for HSPs and amputation induced proteins in the epimorphic regeneration of the amphibian limb.

Acknowledgements

If this
Be but a vain belief, yet, oh! how oft-
In darkness and amid the many shapes
Of joyless daylight; when the fretful stir
Unprofitable, and the fever of the world,
Have hung upon the beating of my heart-
How oft, in spirit, have I turned to thee,
O sylvan Wye! thou wanderer thro' the woods
How often has my spirit turned to thee!

-Tintern Abbey-
W. Wordsworth, 1798

When I heard the learn'd astronomer,
When the proofs, the figures, were ranged in columns before me,
When I was shown the charts and diagrams, to add, divide and measure them,
When I sitting heard the astronomer where he lectured with much applause in the lecture-room,
How soon unaccountable I became tired and sick,
Till rising and gliding out I wander'd off by myself,
In the mystical moist night-air, and from time to time,
Look'd up in perfect silence at the stars.

W. Whitman

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Introduction

Amputation of a urodele forelimb initiates a series of developmental events which lead to the restoration of the missing portion (Tassava *et.al*, 1987). Thus epimorphic regeneration is a powerful system in which to study these cellular/molecular mechanisms underlying this process. In response to amputation, three distinct, nerve-dependent (Schotte and Butler, 1944) phases of regeneration occur. Firstly, there is an accumulation of undifferentiated cells distally (the preblastemic phase), followed by proliferation of these mesenchymal cells (the blastemic phase) with subsequent morphogenesis or redifferentiation of cells into a mature, functional limb (Wallace, 1981; Liversage, 1987).

The influences on the process of epimorphic regeneration of the limb are many. For example, since the 1970s, it has been known that a diffusable neurotrophic factor, probably a polypeptide(s), distinct from neurotransmitters, was released from the truncated nerves in the limb stump (Dearlove and Stocum, 1974; Singer *et.al*, 1976; Choo *et.al*, 1978; Singer, 1978; Mescher and Gospodarowicz, 1979; Carlone *et.al*, 1981; Fallon and Caplan, 1983; Brockes, 1984; Carlone and Rathbone, 1985; Hopkins and Hughes, 1985; Bao *et.al*, 1986; Brockes and Kintner, 1986; Landesman and Copeland, 1988). There are also stage-dependent qualitative and quantitative differences in the soluble protein content of adult newt forelimb regenerates (Schmidt, 1968; Dearlove and Stocum, 1974; Slack, 1982; Garling and Tassava, 1984; Bao *et.al*, 1986) that exert influences on the regenerating blastema.

Appropriately, therefore, some studies have been designed to characterize and elucidate the roles of the regeneration-unique proteins involved in the special growth and differentiation needs of a developing blastema (Schmidt, 1968; Dearlove and Stocum, 1974; Slack, 1982; Garling and Tassava) or the transient state of cellular metaplastic transition (Wier

and Scott, 1986a; 1986b; 1987). One biochemical process that has not been examined in the regenerating forelimbs of the newt is the heat shock response.

The heat shock response is ubiquitous and is characterized by the suppression of the synthesis of cellular proteins and the selective synthesis of highly conserved set of heat shock proteins (HSPs), (Ashburner and Bonner, 1979; Burdon, 1986; Subjeck and Shyy, 1986; Lindquist and Craig, 1988). Three major families of HSPs, categorized by their molecular weights, HSP83-like, HSP70-like and the small HSPs (15-30kDa) are synthesized in response to a number of environmental stresses (Burdon, 1986; Pelham, 1986; Lindquist and Craig, 1988) and appear to play a role in cellular protection during aberrant environmental conditions.

Although the function and mechanism of action of the heat shock proteins remains unclear, recent evidence suggests that the expression of at least some of the members of the major HSP families is not only regulated in response to stress conditions but also during the normal embryogenesis of some organisms. This implies a role for these HSPs in development under normal physiological conditions (Morange *et.al*, 1984; Heikkila *et.al*, 1985; Heikkila *et.al*, 1986; Palter *et.al*, 1986; Bonato *et.al*, 1987; Lindquist and Craig, 1988).

The developmental regulation of HSPs, the demonstration of their induction in response to wounding (Currie and White, 1981; Heikkila and Schultz, 1984) and the recent interest in their possible roles in mitogen stimulation (Wu and Morimoto, 1985; Ferris *et.al*, 1988) and retinoic acid induced differentiation (Bensaude and Morange, 1983; Imperiale *et.al*, 1984) of cultured cells, has led us to examine their expression in the regenerating forelimbs of the newt. Specifically, we have analyzed HSP70(s) synthesis and accumulation by fluorography and Western blotting in stump tissues of the regenerating forelimb of the newt in response to the stress of hyperthermia,

and amputation. We have also examined its normal expression at specific regeneration stages corresponding to periods of dedifferentiation, proliferation and redifferentiation of the blastema cells.

The results may enable a clearer understanding of the role played by amputation induced proteins and developmentally regulated heat shock proteins during the epimorphic regeneration of the amphibian limb. Some of the implications of this thesis have been discussed previously by Carlone and Fraser (1989).

Literature Review

Amphibian Limb Regeneration

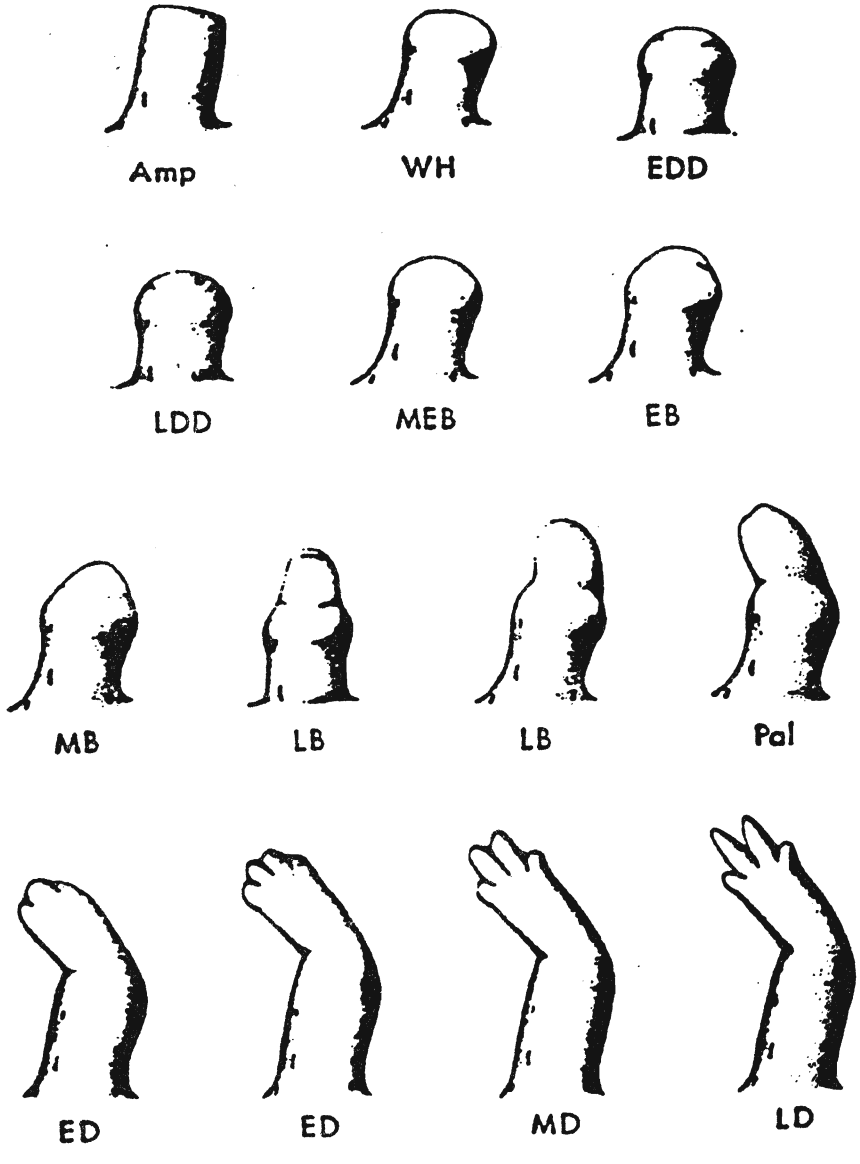
The term regeneration has been used to describe replacement of appendages in vertebrates and invertebrates, repair of tissue such as muscle and skin, and restoration of visceral organs after their wounding or partial removal. There are generally two types of regeneration, i.e., tissue and epimorphic. Tissue regeneration is simply the replacement of lost cells and extracellular material by the remaining cells. Epimorphic regeneration, on the other hand, is the type found in regenerating appendages. There are at least eight requirements for epimorphic regeneration: injury, adequate innervation, the presence of bioelectric fields, wound epidermis, a contribution from the immune system, a source of undifferentiated cells, the hormonal milieu and the spatial/morphogenetic information (Tassava and Loyd, 1977; Sicard, 1985; McGinnis and Vanable, 1986; Liversage, 1987).

The process of epimorphic regeneration is best exemplified in vertebrates by the regeneration of the amphibian limb, the lizard tail, and the fish fin. Vertebrates show a variability in limb regenerative potential; the best example of complete regeneration is found in the adult urodele amphibian, *Notophthalmus viridescens*. A few anuran species such as *Xenopus laevis*, are capable of only heteromorphic limb regeneration while reptiles have very restricted capacity of limb regeneration. Though most vertebrate regeneration is limited, there are fundamental pathways (i.e. epithelialization) that are common even between such diverse phyla as mammals and urodeles (Donaldson and Mason, 1975; Reichman, 1984; Liversage, 1987).

Figure I. Drawing of dorsal view of a right limb amputated through the distal third of the humerus progressing through the epimorphic stages of regeneration. The abbreviations for each stage are:

Amp= limb stump immediately after amputation
WH= wound healing
EDD= early dedifferentiation
LDD= late dedifferentiation
MEB= moderate early bud
EB= early bud
MB= medium bud
LB= late bud
Pal= palette
ED= early digits
MD= medium digits
LD= late digits

Figure III. Chronological Morphology of Limb Regeneration



(Iten and Bryant, 1973)

The ability of the North American red-spotted newt, *Notophthalmus viridescens* to regenerate a lost limb or tail has been studied extensively and the external morphology of the regenerating forelimb has been well documented (Figure 1). Following amputation of the newt forelimb, epidermal cells migrate over a fibrin clot at the wound surface to form an apical cap of thickened epithelium (wound epithelium) followed by a fairly typical inflammatory reaction. The first events, therefore, are those associated with wound healing. The manner in which these events proceed is crucial in determining whether or not regeneration actually occurs. Typically, following amputation, urodele wound healing proceeds without cicatrization, the formation of fibrous tissue deposits; however, should a cicatrix form due to denervation, all further signs of regeneration fail to appear (Wallace, 1981; Sicard, 1985).

As wound healing progresses, a growth zone composed of histologically undifferentiated cells is visible beneath the wound epithelium (Wallace, 1981). These mesenchymal cells form the blastema and represent the progenitor cells of the regenerate. Once a critical mass of blastema cells accumulates within the regenerate the cells undergo cytodifferentiation and morphogenesis, reforming the physiologically correct limb (Singer, 1974). Thus at the cellular level, there are three distinct phases of regeneration that include firstly, accumulation of undifferentiated cells (preblastemic), followed by proliferation of these mesenchymal undifferentiated cells (blastemic) and finally, the morphogenesis or redifferentiation of cells into a mature, functional limb (Wallace, 1981). The chronological sequence of characteristic stages and their influencing regulators have been standardized for the purpose of comparison (Table I). Though the process of regeneration

Table 1. Stage and Regulatory Influences of Urodele Limb Regeneration

Phase	Stage	Time in days (20°C)	Regulatory Influences: Electical	Endocrine	Nerves	Immune	
Preblastemic accumulation of undifferentiated cells	Wound healing	0-1	+(?)*	+	+	+/-(?)	
	Early dedifferentiation	7	+(?)	+(?)	+	+/-(?)	
	Late dedifferentiation	13	+(?)	+(?)	+	+/-(?)	
Blastemic proliferation and growth	Moderate early bud	15		+	+	+/-(?)	
	Early bud	19-21		+	+		
	Medium bud	21-25		+	+		
Redifferentiation and morphogenesis	Late bud (cone)	25-30		+	+/-(?)		
	Palette (paddle)	31-35		+	+/-(?)		
	Early digit	37-40		+	+/-(?)		
	Medium digit	43-50		+	+/-(?)		
	Late digit	48-		+	+/-(?)	-(?)	

*+ indicates regulator acts to promote particular event or activity
- indicates regulator acts to prevent or delay event or activity
+/- denotes an effect that might either promote or adversely affect the event
(?) indicates a proposed or suggested role, no influence on this event has been demonstrated

(Wallace, 1981; Sicard, 1985)

is constant, the rate of regeneration is temperature dependent (Wallace, 1981).

Blood Clotting/Wound Epithelialization

Initial responses to trauma are reflected in the tissues during approximately the first twenty-four hours after amputation through the forelimb. During regeneration of the forelimb there is an intimate association between the vascular system and the blastema. This network of blood vessels serves as the channels through which various factors and leukocytes are conveyed to the blastemal cells (Sicard, 1985).

Unlike mammals, when the whole forelimb of a urodele is amputated the flow from major brachial arteries is rapidly reduced, and the total blood loss appears negligible. Clotted blood at the amputation surface serves to close off the gaping wound from the external environment. The clotting process in amphibians is extremely rapid when compared with clotting in mammals and is characterized by a lattice work of fibrin at the wound surface. In adult urodeles, clotting time is between two and three minutes. The mechanism of clotting in urodeles is still unknown, as is its immune system, but appears to parallel that of mammals though of lower sophistication (Schmidt, 1968).

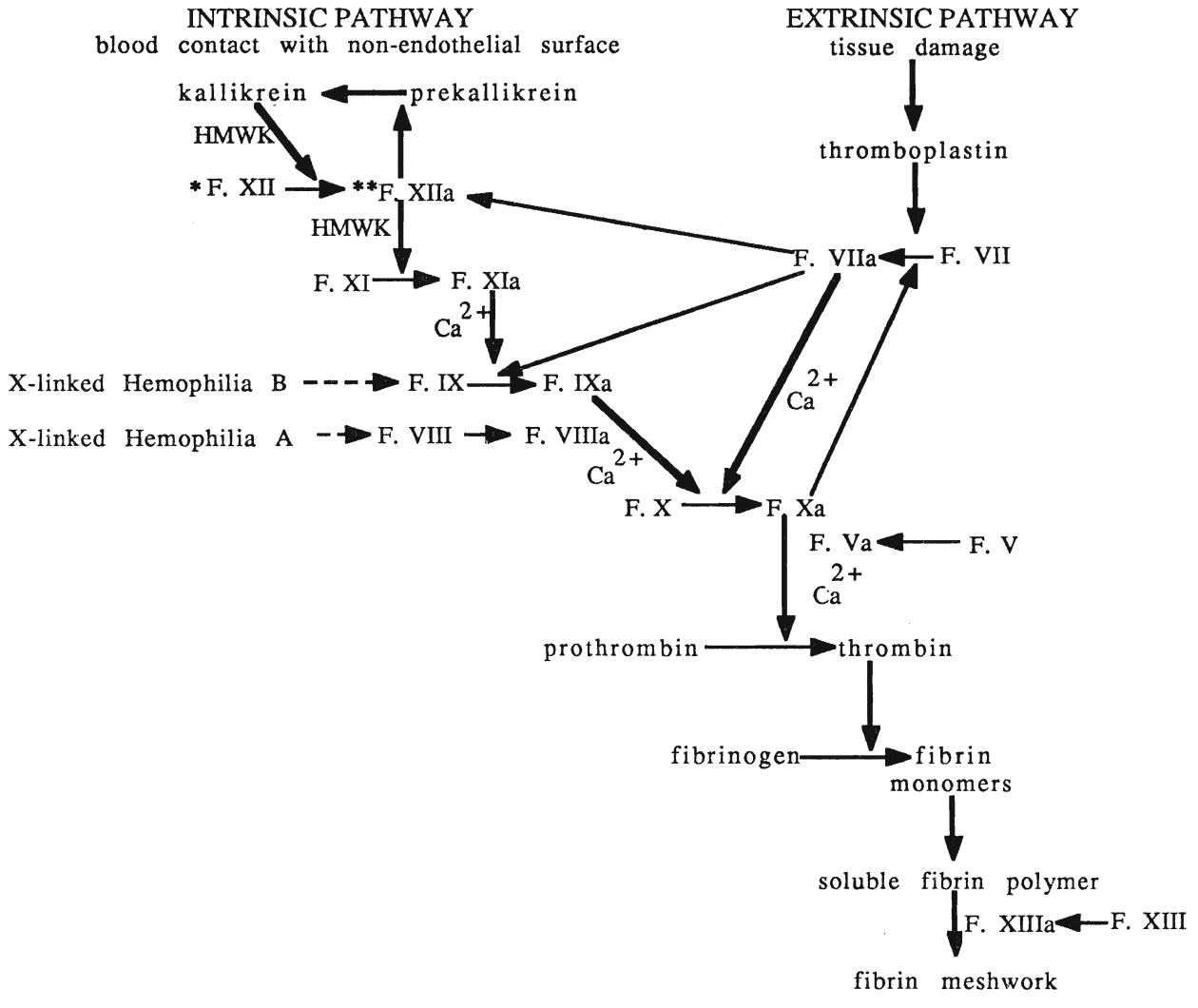
Mammalian haemostasis is a collective term for the cessation of blood flow through a damaged vessel wall by the coordinated action of platelets, clotting factors, endothelial cells and vessel musculature. When mammalian fluid exudes form the blood stream into the tissues, three enzyme cascades are activated: the complement system; the coagulation system; and the fibrinolytic system. These interacting reactions are characterized by the activation of serine protease class enzymes that are stable in an inactivate zymogen form (Tizzard, 1988).

In mammals, vasoconstrictive agents such as serotonin (5-hydroxytryptamine) and kinins are released by platelets and may be an effective process to stop bleeding in the capillary bed, but is not sufficient for haemostasis in arterioles and venules. In these vessels the critical step is the immediate reaction of the blood platelets with the subendothelial structures. The activation of blood platelets and their morphological change is by a number of cellular responses. Among these stimulators are collagen, epinephrine, platelet-activating factor, immune complexes, serotonin and the coagulation system's multifunctional enzyme thrombin (32kDa). The platelets release a number of clotting factors, i.e. von Willebrand factor (high mol. weight), fibrinogen (340kDa, polymer), factor V (330kDa), and thromboxane A_2. These releasing factors influence platelet stimulation, aggregation and adhesion with subendothelium. In addition, the reversible binding of the cofactor fibrogen, factor V and the thrombin-activated factor V_a to the platelet membrane serve as membrane receptors for coagulation factors. This close cooperation between platelets and clotting factors results in the production of a fibrin-reinforced platelet plug localized at the site of the vascular defect (Figure II). Apart form this, platelets participate in a number of other physiological reactions, such as phagocytosis, inflammation, immunological reactions, and interactions with tumour cells (Zwaal and Hemker, 1986).

Platelets and their subsequent haemostatic effects with a fibrin matrix are not the first trigger for the activation of the coagulation cascade. Blood clotting starts when mammalian tissues are injured and a polypeptide component, Hageman factor (factor XII), is liberated and activated. This initiating factor leads to a series of enzyme reactions that result in large quantities of thrombin, the main clotting enzyme, being formed from

Figure II. A schematic representation of the coagulation cascade reaction in mammals. The bold lines represent the classical division of the coagulation cascade in an intrinsic and extrinsic pathway. The connecting lines show points of interaction between both pathways.

Figure II. Mammalian Coagulation Cascade System



* F. XII designates inactive zymogen form of factor XII
** F. XIIa designates active form of factor XII
HMWK, high molecular weight kininogen

(Zwaal and Hemker, 1986)

prothrombin (70kDa). Thrombin acts on the fibrinogen in tissue fluid and plasma to produce insoluble strands of fibrin (polymer of low mol. weight monomers). This irregular matrix of fibrin is laid down in inflamed tissues and capillaries and forms the substratum for epidermal migration as well as an effective barrier to the spread of infection. The collagen-induced activation of factor XII_a (28kDa) seems less important for the cessation of traumatic bleeding (Zwaal and Hemker, 1986; Tizzard, 1988).

The ordered and controlled interplay of the coagulation cascade reactions (Figure II) is accomplished by the high degree of specificity of the coagulation enzymes in positive and negative feedback mechanisms. The blood coagulation system in humans can be activated by two pathways: 1) the extrinsic pathway of tissue damage or 2) the intrinsic pathway which is moderated by negatively charged materials such as glass, celite, pyrophosphate endotoxin and certain connective or collagen preparations. Present evidence suggest that the coagulation process is both autocatalytic and self-limiting and that thrombin plays a central role. The active coagulation enzymes can only exist at the site of injury for a short time, whilst the inhibitors of these proteolytic enzymes are present in the whole vascular system (Zwaal and Hemker, 1986). Adding to the complexity of the coagulating system is the role of vitamin K-dependent protein C (62kDa, dimer). It has been suggested the activated protein C not only has a local regulating function in blood coagulation but that it also has a systemic anticoagulant effect and is involved in fibrinolysis of the fibrin matrix (Zwaal and Hemker, 1986).

In mammalian systems, the control of the clotting process by the digestion of the fibrin lattice work is due to plasmin (92kDa, dimer), a potent fibrolytic enzyme. Plasmin is activated from inactive zymogen form,

plasminogen, by the Hageman Factor (74-80kDa). In destroying fibrin, plasmin releases peptide fragments that are chemotactic for neutrophils and thus the inflammatory response (Tizzard, 1988).

In contrast to the non-nucleated platelets, non-mammalian vertebrates (e.g. newts) possess a special class of nucleated cell, the thrombocyte, that activates the clotting process. These cells can be stimulated by thrombin and collagen. Platelet cells are actually the evolutionary progeny of the stem cell-thrombocyte system that evolved into the stem cell-megakaryocyte system found in mammals and humans. To date, little is known about thrombocytes (Sicard, 1985, Zwaal and Hemker, 1986).

Within the next nine hours after the fibrin plasma clot forms over the surface of the amputation, a wound epithelium lacking any underlying dermis or basement membrane forms. Normal amphibian skin tissue is composed of the epidermis which consists of an outer covering of keratinized cells called the stratum corneum. This layer of cells differentiates from proliferating inner cuboidal cells termed stratum germination. Superficial to the stratum germination is the basement membrane. The basement membrane contains two components, a basal lamina and the subjacent reticular lamina. In addition to collagen, the basement membrane has been shown to contain fibronectin, reticulin and laminin. Beneath this is the dermis, whose main representative cell type is the fibroblast. It is in the dermis that large glands derived from the epidermis are located (Sicard, 1985).

In mammals, epidermal cell-derived factors (EDF) and transforming growth factor-α (TGF-α) play major roles in both proliferation and migration of keratinocytes that differentiate into epidermis thereby accelerating

epidermal regeneration (Eisinger *et.al*, 1987; Shultz *et.al*, 1987). The role of these epidermal growth factors during the formation of the newt wound epithelium has not been investigated to date.

There is no doubt that the events leading to reconstruction of the newt limb begin with epithelialization. It was demonstrated by experimentally labeling cycling populations of the epidermis with [^{3}H]thymidine before amputation and following their migration after amputation, that the wound epithelium is closed by the distal migration of the retracted stump epithelium. These migrating epidermal cells have characteristically normal differentiation products (i.e. mucous granules, tonofilaments), though they have an elongated morphology and contain a reduction of desmosomal contacts with each other and with the adepidermal reticulum. Epithelialization of the wound plane and the increased presence of intracellular microtubules are not inhibited by protein synthesis as they are unaffected by cycloheximide incubation. The microtubule bundles have been suggested to be involved with cellular locomotion and contact guidance (Schmidt, 1968; Repesh and Oberpriller, 1978, 1980; Tassava *et.al*, 1987; Eisinger *et.al*, 1988). The general morphology and inter/intracellular characteristics of migrating epidermal cells is consistent in other systems where translocation is known to occur (Hirobe, 1983, 1984, 1988).

Electron microscopy (SEM, TEM) of the ultra-structure of the epidermal cells which migrate over the wound surface of the amputated newt limb was conducted by Repesh and Oberpriller (1978, 1980). They observed that at one hour post-amputation, a smooth and moist fibrin covered wound surface in which the underlying stump tissues were no longer discernable. Epidermal migration was just initiating as its progress was restricted to the periphery of the wound. Occasional blood cells were

observed on the wound surface and near the site of amputation. The apparent onset of migration of epidermal cells was observed at approximately two to five hours post-amputation. From the epidermal cut edge, a single layer of basal and suprabasal cells detach from the basal lamina and encroach by the extension of pseudopodia upon the fibrin-covered wound surface. This early onset of epidermal motility is in contrast to the slower mammalian epidermal migration which commences between approximately twelve to twenty-four hours after wounding. The duration of mammalian wound healing also diminishes with the developmental age of the organism (Repesh and Oberpriller, 1980; Hirobe, 1988).

By nine hours post-amputation, the epithelialization of the wound surface is complete. The irregular alignment of the fibrin clot wound architecture appears to be a necessary requirement for the epidermal locomotion. This filamentous scaffolding, derived form the clotting of blood, acts as a substratum (an extracellular matrix) facilitating epidermal cells' interaction and thus migration. Staining of the wound fibrin surface with the cationic stain, RR, revealed the presence of covalently bound carbohydrate-containing material. It has been speculated that this material could conceivably contain fibronectins which play a role in cellular motility. Fibronectins are a family of glycoproteins that are involved primarily with the adhesion of cells to one another or to substrata. The soluble plasma form, globulin, has a marked affinity for fibrin and fibrinogen and has been found to be covalently incorporated into fibrin clots (Repesh and Oberpriller, 1978, 1980).

Histological evidence demonstrated that dispersed within the advancing epidermal sheet were cells of apparent hematogenic origin. These included erythrocytes, phagocytic leukocytes (chiefly granulocytes) and

melanocytes of various stages of melanin production. During the first few days of the inflammatory response, neutrophils are the predominant cells associated with wounds. These cells, containing powerful hydrolytic enzymes, were consistently adjacent to the fibrin exudate of the wound and participate in the demolition phase of wound healing (Repesh and Oberpriller, 1978, 1980; Schmidt, 1968). Removal of extracellular matrix, damaged cells and intracellular material by the neutrophils is assisted by other leukocytes (e.g. phagocytic macrophages and lymphocytes) as the inflammatory response progresses to day five (Sicard, 1985).

Continued epidermal migration causes the wound epithelium to thicken into an apical epidermal cap (AEC), detectable by day four. In addition, local edema may accumulate in the AEC giving the stump a distended appearance (Wallace, 1981). Work by Tassava *et.al.* (1987) studying the reactivity of a monoclonal antibody, WE-3, have suggested that the early wound epithelium is gradually replaced by cells from the dermal skin glands during blastema outgrowth. This reorganization of the wound area and the composition of the extracellular matrix is moderated by the presence of macrophages and lymphocytes which stimulate fibroblast proliferation and collagen synthesis. The mechanism by which these two classes of leukocytes induce this change and the removal of the fibrotic scar is still undetermined (Sicard, 1985).

The amphibian wound epithelium and subsequent AEC lack any underlying dermis or distinct basement membrane. This intimate and essential contact with the underlying mesodermal tissues and later with the blastemal mesenchymal-like cell is required until the start to blastemal redifferentiate, i.e., the late bud stage (Wallace, 1981). In Contrast to the amphibian wound epithelium which lacks a reconstituted basement

membrane, in other vertebrate limbs after amputation, a simple wound healing response is the sole extent of the repair process. In such cases, the epidermis moves through the dry fibrous tissue of the dermis, beneath the exudate to form the scab, i.e. cicatrix. The existence of the cicatrix is reputedly sufficient to inhibit the morphological regeneration (Repesh and Oberpriller, 1980; Sicard, 1985).

A functional wound epithelium must be present over the amputation surface for blastemal development and regeneration to occur. Experimental procedures that adversely affect the formation or function of the wound epithelium are detrimental to regeneration. These include insertion of freshly amputated limbs into the body cavity to prevent epidermal healing, frequent surgical removal of the wound epidermis as it reforms and covering of the wound surface by an allograft of whole skin (Tassava and Loyd, 1977; Tassava and Olsen, 1982; Garling and Tassava, 1984). In addition, the apical epidermal cap appears to be active in maintaining the undifferentiated state of the blastemal mesenchyme subjacent to it, an idea compatible with the proximodistal pattern of redifferentiation within the limb (Tassava and Mescher, 1975; Tassava and Loyd, 1977; Sicard, 1985). As the blastema increases in volume, the proximal cells, which are no longer in contact with the AEC, are the first cells to develop tissue specific characteristics (Stocum and Dearlove, 1972; Carlson, 1975; Tassava and Mescher, 1975; Wallace, 1981). A number of other functions have been proposed for the AEC, however, none are well documented.

Nerves/Neurotrophic Growth Factors

The phenomenon of adult newt forelimb regeneration was shown to be nerve dependent by Tweedy John Todd in 1823. Early research established that the three distinct cellular phases of regeneration (i.e. wound

epithelialization and accumulation of undifferentiated cells, proliferation and redifferentiation) are all nerve dependent (Schotte and Butler, 1944). More recently it has been shown that it is not the mere presence of nerve but rather the quantity of limb innervation (15 fibres/0.01mm^2 limb cross-sectional area) that determines whether an amputated limb regenerates (Singer, 1974, Wallace, 1981). Hence, regeneration in the newt requires a nerve supply, a fact known as the neurotrophic phenomenon (Singer, 1965; Wallace, 1981).

If the limb is deprived of innervation (e.g. brachial nerves III, IV and V) or innervation is blocked by the administration of colchicine immediately before or during the wound healing phase, reepithelialization and phagocytosis proceed but no limb bud is formed (Wallace, 1981; Scadding, 1988). Reminiscent of the mammalian wound healing, denervation at this time causes the formation of a cap of cartilage at the tip of the stump covered by a thick cicatrix (Dearlove and Stocum, 1974; Choo *et.al*, 1978; Liversage and McLaughlins, 1983). When the limb is denervated during the undifferentiated accumulation and proliferative phases (prior to the late bud stage) one observes the attenuation of cellular division after a six to eight hour lag-phase in which the rates of RNA, DNA and protein synthesis decreases. Subsequently, the denervated blastema undergoes the onset of atrophy and resorption (Wallace, 1981).

Evidence for nerves acting in both the G_1 and G_2 phase of the cell cycle has accumulated. Though their precise influence in the cell cycle phase is debatable a major role for G_1 is suggested due to blockage at this phase in denervated limbs. Thus nerves were deduced to exert a mitogenic effect, as judged by [^{3}H]thymidine incorporation, on the blastemal cells (Bryant *et.al*, 1971; Tassava and Mescher, 1975; Singer *et.al*, 1976; Singer, 1978; Fallon

and Caplan, 1983). Mitotic arrest can be reversed by the infusion of neural tissue homogenates directly into the denervated limb bud *in vivo* and *in vitro* or the surgical grafting of dorsal root ganglia implants. This reversal is extremely time specific. A delay of twenty-four hours between denervation and reinnervation significantly reduces the responsiveness of the regenerate (Singer *et.al*, 1976; Choo *et.al*, 1978; Wallace, 1981; Tomlinson and Tassava, 1987). In contrast, denervation after the onset of redifferentiation (subsequent to the formation of the late bud stage), results in an anatomically complete but smaller than normal regenerate due to the interruption of a complete critical mass of cells (Wallace, 1981, Maier *et.al*, 1984; Sicard, 1985).

Since the 1970s, many investigations into the nature of the neurotrophic phenomenon in urodele regeneration have been made. Early results indicated that a diffusable neurochemical, distinct from neurotransmitters, was released from the nerves. A decrease of radiolabelled protein synthesis in the denervated regenerate (Dearlove and Stocum, 1974; Singer *et.al*, 1976) and the observation (Choo *et.al*, 1978) that heating and protease treatment destroy the mitogenic activity of previously active neural extract indicate that the putative neurotrophic agent(s) is a protein. These factors that influence the mitotic index of cells and their rate of protein synthesis are termed growth factors. They constantly appear to be basic proteins of relatively low molecular weight (Singer *et.al*, 1976; Singer, 1978; Mescher and Gospodarowicz, 1979; Carlone *et.al*, 1981). In the neuron, only the cell body contains all the cellular machinery needed to synthesize proteins. Protein synthesis in the cell body can be transported via both fast and slow transports (Wallace, 1981; Sicard, 1985). Singer (1974), however,

revealed that the neurotrophic agent(s) does not arise from the Schwann cells of the myelinated nerve fibres.

To help elucidate the neural growth factor(s) that effects newt regeneration, many investigators have taken advantage of Singer's (1974) evidence that the factor(s) is not species-specific and is present in both nerves and brain (Singer *et.al*, 1976; Choo *et.al*, 1978). Rapid and simple *in vitro* and *in vivo* bioassays have been developed to test growth factors that have been purified from higher vertebrates on blastemal cell cultures. It has been suggested that any candidate for a neural mitogen in the regeneration of the newt should satisfy four criteria (Brockes, 1984). These include: 1) the putative factor should be present in the blastema, 2) be lost upon denervation of the forelimb, 3) be able to stimulate the division of those blastemal cells whose proliferation is normally dependent on the nerve and 4) lose all biological activity, mimicking the denervation state, when an antibody to the factor is introduced into the normally innervated blastema. In addition, similar bioassays have been used to test the effects of growth factors on other homogeneous cell populations (Choo *et.al*, 1978; Singer, 1978; Carlone and Foret, 1979).

By use of this technique, a number of potential brain and neural growth factors have been identified and characterized. Although no one factor which unequivocably govern's limb regeneration in the adult newt has been found, recent convincing evidence supports a role for the nerve-dependent Glial growth factor (Brockes and Kintner, 1986) and the pituitary growth hormone (Landesman and Copeland, 1988) in contributing to blastemal proliferation. Finally, it has been proposed that a combination of several neural growth factors interact (i.e. negative and positive growth regulation) with other molecules to influence the epimorphic regeneration of

a blastema (Choo *et.al*,1978; Singer, 1978; Carlone and Foret, 1979; Fallon and Caplan, 1983; Brockes, 1984; Mescher and Munaim, 1984; Carlone and Rathbone, 1985; Hopkins and Hughes, 1985; Bao *et.al*, 1986; Brockes and Kintner, 1986; Boilly and Bauduin, 1988).

Dedifferentiation

As mentioned previously, a key event in amphibian regeneration is the formation of new tissue, at the stump end, known as the undifferentiated, pluripotent blastema. It has been suggested that the cells of the blastema arise after wound healing by local dedifferentiation of the mature tissues of the limb, including muscle, cartilage, connective tissue and Schwann cells. Thus, the morphogenesis of the amphibian limb regenerate may involve to a significant degree cellular trans-differentiation or metaplasia. Dedifferentiation is the process whereby differentiated cells lose their cell specific characteristics and take on those of undifferentiated precursor cells. It is generally accepted however, that the epidermis does not contribute to the blastemal mass (Wallace, 1981).

Dedifferentiation of myofibres has been speculated to occur after amputation, although proof has been inconclusive since muscle contains a variety of cell types (Kintner and Brockes, 1984; Griffin *et.al*, 1987; Gordon and Brockes, 1988). Skeletal muscle of adult, metamorphosed urodele amphibians, lack myogenic reserve cells (satellite cells) that are present in the larval urodele. There is indication that they contain cells, termed post-satellite cells, that function as a myogenic reserve source (Cameron *et.al*, 1986). Hence, studies of myofibres after amputation have lead to confusion regarding the source of the undifferentiated mesenchymal blastema cells; whether they are derived from tissue dedifferentiation or a stem cell-like reserve population.

Kintner and Brockes (1984) used tissue specific monoclonal antibodies (e.g. 22/18 and 12/101) to try and address the question of the precursor blastema cell source. Their rationale was that if one cell type is derived from another, then a proportion of dedifferentiated cells might show an intermediate phenotype and label with both cell markers in the blastema. Interpretation of their results indicate that the blastema is made up of a heterogeneous population of morphologically similar cells and not a homogeneous population as previously thought (Wallace, 1981). Determination of the origin for these cells is still controversial as double labelling of blastema cells and post-satellite cells has been observed (Cameron *et.al*, 1986; Griffin *et.al*, 1987; Gordon and Brockes, 1988).

Immunohistochemistry has some disadvantages, such as quantitative cell analysis, as well as inherent technical artefacts. In order to circumvent these problems, Casimir *et.al* (1988) have utilized tissue specific gene methylation patterns as a molecular marker to follow cell fates during limb regeneration. Preliminary data utilizing this lineage marker technique indicates dedifferentiation and metaplasia occurs in amphibian limb regeneration. The validity of the results is not contested though certain caveats must be acknowledged due to the intrinsic lack of cellular resolution (Casimir *et.al*, 1988).

To date, the issue of the blastemal cells' origin has not been resolved; dedifferentiation and activation of post-satellite cells from one or several tissues are both viable and perhaps not mutually exclusive (Wallace, 1981; Sicard, 1985). The refinement and increased resolution of methylation lineage markers will hopefully enable the determination of the mechanism by which the progenitor blastemal cells arise in the very near future.

Proliferation

The proliferation and accumulation of a critical mass of blastema cells at the site of limb amputation is required for limb regeneration to ensure. Tassava and Mescher (1975) proposed a model in which injury stimulates quiescent differentiated cells of the stump tissue in the G_0 (gap) phase to dedifferentiate and re-enter the cell cycle at the G_1 phase. The cells then pass through the DNA synthesis (S) phase in which the cell doubles its DNA content (4N), then a second gap phase, G_2, and then finally undergo mitosis during the M phase. The cell cycle time of the blastema cells is approximately 40-50 h in length with a S period of 60 to 80% of this time. They proposed that the proliferative state is due to the tripartite control of the injury itself, the AEC and the nerves (i.e. neural growth factor). To date, it is known that the cellular interaction which elicits the proliferation phase of amphibian regeneration is also regulated by hormones (e.g. insulin) and possibly calcium and cyclic nucleotides (Fallop and Caplan, 1983; Sicard, 1985; Globus *et.al*, 1987).

It was generally accepted that the newt blastema (i.e. moderate early bud to medium bud) was composed of proliferating cells that are asynchronously distributed about the cell cycle and actively progressing toward their next mitosis (Wallace, 1981). This assumption has recently been disproven by combining continuous and pulse-chase tritiated thymidine labelling. At any given moment, only 26% of the blastema cells are actively progressing through the cell cycle, with the remainder being in a state of transient quiescence. It has been suggested that nerves may be primarily involved in the entry of quiescent cells into the active cell cycle population. Denervation paralleled control mitotic index values for forty-eight hours following nerve withdrawal. Subsequently, the mitotic index fell sharply and

was essentially zero for cell cycle activity. The failure of denervated limbs to regenerate was correlated with an increased non-proliferative fraction and a reduced proportion of actively cycling cells (Goldhamer and Tassava, 1987; Tassava *et.al*, 1987; Tomlinson and Barger, 1987).

Between the late bud and palette stage the blastema attains a critical cell mass preventing further 'dedifferentiation' of stump tissue. At this period, while the cells of the regenerate are proliferating and the blastema continually increasing in volume, a wave of redifferentiation begins at the stump and proceeds in a distal fashion such that the digits are the last to form (Schmidt,1968; Singer, 1978; Wallace, 1981).

Redifferentiation

Two divergent models: the polar coordinate and the zone of polarizing activity, have been postulated for explaining how patterning occurs in the vertebrate limb (Bryant and Muneoka, 1986). In general, mesenchymal cells of the amphibian limb regeneration blastema inherit from their parent limb cells a memory of their level of origin. This positional memory or value specifies the proximal boundary of the regenerate by preventing blastema cells forming structures proximal to their level of origin. Since the limb regenerates exactly what is amputated from any level, its proximodistal axis can be viewed as a series of such potential regenerate boundaries. Similar sets of level specific memories appear to exist along the anteroposterior and dorsoventral axes of the limb. The AEC serves to provide information about the external boundary of the regenerate, while the dermis plays the more major role, controlling the form and asymmetry of the regenerate (Stocum and Dearlove, 1972; Carlson, 1975; Sicard, 1985; Crawford and Stocum, 1988; Newman, 1988).

Interestingly, Sessions *et.al.* (1989) have inferred, form results from limb bud allografts, that urodeles (e.g. the axolotls) and anurans (e.g. *Xenopus*) share the same patterning mechanism. Thus this common, basic patterning mechanism is apparently ancient, and appears to be based on a fundamental property of cell interaction that is characteristic of epimorphic systems.

Stage-Dependent Protein Synthesis

In contrast to the many quantitative studies on the effects of denervation on blastema protein synthesis in urodele amphibians, few attempts to determine quantitative and/or qualitative patterns of macromolecular synthesis in innervated blastemal forelimb regenerates have been completed (Dearlove and Stocum, 1974; Slack, 1982; Garling and Tassava, 1984). In addition, the earliest previous characterization of the blastema protein synthetic pattern was obtained at three days post-amputation (Garling and Tassava, 1984).

Dearlove and Stocum (1974) examined the low molecular weight proteins of innervated blastemas by SDS-polyacrylamide tube gel electrophoresis, and found their results to be in agreement with those first obtained by Schmidt (1968). Coomassie blue staining of the tube gels revealed that the protein composition of the regenerate changes from stage to stage of its development. Eight new proteins unique to the regenerating limb were observed in addition to the twenty-four distinct proteins bands in the unamputated controls. Although the eight regeneration-unique proteins were not characterized, five of the eight appeared at day ten post-amputation. At this stage, 'dedifferentiation' is well underway and the blastema cells are migrating into the area beneath the wound epidermis and have begun to divide in the stump. These five regeneration-unique proteins

persist at all subsequent stages, except for the early bud stage, where only three are present. By approximately three months post-amputation, the intact limb array of twenty-four proteins was restored (Dearlove and Stocum, 1974).

^{35}S-methionine fluorographs of two-dimensional gels by Slack (1982), revealed differences in peptide synthesis associated with differentiating regions of regenerates and between blastemal mesenchyme and wound epidermis in the forelimbs of the axolotl. Interestingly, the protein synthetic pattern in mesenchyme from the larval limb bud was almost identical to that in the medium bud mesenchyme. The medium bud mesenchyme differs from the unamputated mesenchyme by the specific synthesis of seven proteins. At the medium bud stage of regeneration, thirteen epidermal specific proteins were present with ten of them being covalently modified by phosphorylation. Since these thirteen spots were also present in limb skin, their role in the function of wound epidermis is unclear. Although their identities were not unambigously determined, Slack postulated that they were probably keratins due to their molecular weights (65, 63, 59, 55, 50, and 39 kilodaltons).

The two-dimensional protein patterns for the late bud stage epidermis and mesenchyme were similar to the patterns found at the medium bud stage with the exception of the appearances of three newly synthesized mesenchymal basic proteins (MW= 60-67kDa). At this stage, dedifferentiation has ceased and redifferentiation has been initiated in the proximal portion of the regenerate. Slack (1982) stated that during the phase of blastemal proliferation, there was little change in terms of protein synthesis pattern in either the epidermis or mesenchyme. In general, the major biochemical changes observed by Slack were associated with the early digit

stage regenerate; a period of cytodifferentiation. At this stage, the muscle pattern resembles the blastema mesenchyme more than the cartilage but both are clearly different from the late bud mesenchyme. The appearance of qualitative protein differences at approximately 35kDa and 80-90kDa were observed in the cartilage while the α-actin, as identified by peptide mapping, was the prominent feature of the muscle pattern with respect to the blastema mesenchyme (Slack, 1982).

SDS-PAGE fluorography by Garling and Tassava (1984), confirmed the results of Dearlove and Stocum (1974) of the presence of 24 bands in the unamputated limbs of the newt. In the regenerates, one band present in unamputated limbs disappeared and four bands (92, 62, 60, 48 and 46kDa, respectively) not present in unamputated limbs, appeared in the blastema. These five qualitative differences in protein synthesis were first detected at 3 days post-amputation in the stump tissue and persisted through the digit stages of regeneration. In contrast to the results of Slack (1982) with two-dimensional fluorography no protein differences were observed by one-dimensional SDS-PAGE fluorography between the wound epidermis and mesodermal core at the early bud stage (Garling and Tassava, 1984).

All of the previous results discussed above are consistent with the idea that the regeneration-unique proteins are involved in the special growth and differentiation needs of a developing system, as well as in the unique process that enables fully differentiated tissues to supply cells to the system by dedifferentiation. Few attempts have been made to identify any of these proteins or to investigate the effects of protein synthesis immediately subsequent to forelimb amputation. Thus, the aim of this present study was characterize the synthesis and possible role of heat shock

proteins (HSPs) and trauma induced proteins in the epimorphic regeneration of the newt limb.

The Heat Shock Response

General Characteristics

Many researchers have recognized that the heat shock response provides a new perspective in which to elucidate some fundamental aspects of cell function, molecular expression and regulation.

All organisms from the simplest prokaryotes (Neidhardt *et.al*, 1984), yeast (Lindquist, 1984; Slater and Craig, 1987), plants (Vierling *et.al*, 1988) on up to higher eukaryotes (Easton *et.al*, 1986; Heikkela *et.al*, 1986, 1987; Mues *et.al*, 1986) have been observed to exhibit similar and reversible cellular responses due to abrupt changes in their environmental circumstances. The rapid induction of this general phenomenon is termed the heat shock response or stress response.

Characteristically, under stress stimulus, select genes which are essentially quiescent under normal physiological conditions, are transiently transcribed and their messages translated into a variety of highly conserved heat shock proteins (HSPs), (Bardwell, 1987; Browder *et.al*, 1987; Chousterman *et.al*, 1987). Concomitant with the preferential translation of the HSPs there is frequently observed a repression of the normal complement of cellular polypeptides via mechanisms of transcriptional and translational controls (Welch, 1985; Burdon, 1986; Lindquist and Craig, 1988).

Although the temporal pattern of HSPs synthesis depends on a number of variables (i.e. organism, developmental stage, cell type, duration and type of stress), the underlying evolutionary conservation of the ubiquitous HSPs suggests that they perform functions that are indispensable to most organisms (Burdon, 1986; Sprang and Brown, 1987). These HSPs have been implicated in several major biological phenomena: embryogenesis

and differentiation, viral infection, growth state, receptor association, metabolism, protection from phenocopy induction and thermotolerance (Subjeck and Shyy, 1986; Simon *et.al*, 1987; Sanchey *et.al*, 1987; Lindquist and Craig, 1988).

Historical Perspective

Polytene chromosomes isolated from *Drosophila* salivary glands had long provided a unique view of transcriptionally active interphase chromosomes. These chromosomes, composed of approximately one thousand aligned chromatids, contain specific regions that undergo a change in conformation during larval development. This process of conformational change is known as puffing due to its extended morphology (Simon *et.al.*, 1985). In 1962, Ritossa first observed by cytological studies that the patterns of puffing sites in the polytene chromosomes of *Drosophila busckii* could be altered upon supra-optimal temperature elevation (Moran *et.al*, 1983).

With the introduction of [^{3}H]uridine autoradiography, the function of the puff sites was elucidated. Specifically, the sites engaged in puffing were shown to be sites of intense RNA synthesis (Spradling *et.al*, 1975) with the kinetics of puff formation being correlated with transcriptional activation of a locus (Simon et.al., 1985). Tissieres *et.al* (1974) and Lindquist *et.al* (1975) reported that temperature elevation caused the dramatic induction of a novel set of polypeptides while repressing the synthesis of the normal complement of proteins as observed by ^{35}S L-methionine incorporation, electrophoresis and autoradiography. The coordinate regression of pre-existing puffs and the appearance of new puff sites presumably reflect the local inactivation and activation of transcription accompanying the change in the cellular protein repertoire.

Isolation and cloning of *Drosophila* HSP genes corresponding to differing molecular weight heat shock proteins were conducted in earnest by Livak *et.al.* (1978). Utilization of these cloned HSP genes and molecular techniques have revealed the ubiquitous nature of the stress response and its highly conserved homology at the nucleotide and amino acid level.

Cellular and Developmental Effects of HSP Inducers

As the name implies, the heat shock response was originally reported to be induced in cells by an elevation of temperature. Since this initial observation, a broad spectrum of agents or physical perturbations (Table II) have been shown to independently or synergistically induce an analogous response (Ashburner and Bonner, 1979; Burdon, 1986; Lindquist and Craig, 1988). Concurrent with the features of the stress response, the inducing agents have been observed to cause a number of cellular and morphological effects (Table II). Some of these cellular effects (e.g. protein denaturation) have been proposed to play a role in the mechanism of HSP induction (Lee *et.al*, 1983; Findley *et.al*, 1984; Burdon, 1986).

Hyperthermia has profound effects on the structural stability of the proteinaceous karyoskeleton (nuclear matrix) assemblies (McConnell *et.al*, 1987). In addition, DNA strand breakage and transient local melting of the DNA duplex occurs at supra-optimal temperature without loss of cell viability due to efficient repair processes (Neidhardt *et.al*, 1984).

A number of inducers such as heat, ethanol, amino acid analogues, heavy metals and thiol reagents may denature, damage, alter configuration or change solubility of some intracellular proteins (Burdon, 1986; Parag *et.al*, 1987). Aberrant proteins may conceivably be formed by increased metabolism and subsequent oxygen-related free radical activity as encountered by glucose-deprivation and the reoxygenation of anoxic cell

Table II. Inducers and Non-Inducers of HSP Synthesis

Inducing agent or treatment	Proposed effects	Non-inducers
Ethanol	Translation errors, AppppN production	Cyclic AMP Amiloride Butyrate
Amino acid analogs, puromycin	Abnormal proteins	Dimethyl sulphoxide Phorbol esters
Heat shock	Increased denaturation	Colchicine Cytochalasin B
Various heavy metals, copper-chelating agents, aresenite, iodoacetamide, p-chloromercuribenzoate, Ca^{2+}	Binding to sulfhydryl grps, conformational changes in proteins	Azacytidine NaCN NaF Dinitrophenol Azide
Return from anoxia, hydrogen peroxide, supeoxide ions and other free radicals	Oxygen toxicity, free radical fragmentation of proteins, AppppN production	Calcium ionophore Cycloheximide Benzocaine
Ammonium chloride,	Inhibition of proteolysis	
Amytal, antimycin, azide, dinitrophenol, rotenone, heptylhyroxy-quinoline N-oxide, ionophores	Inhibition of oxidative phosphorylation, changes in redox state, covalent modifications of proteins	
Hydroxylamine	Cleavage of asparagine-glycine bonds in proteins	
Return from glucose deprivation	Underglycosylation of nascent proteins	
Physical trauma	Cellular disruption and immunological affects	
Pyrogens (e.g. LSD) Ecdysterone Adenovirus E1A protein* Microinjection of denatured proteins Retinoic Acid**		

*induces HSP70-like only
** induces hsp84 and hsp25 only

(Currie and White, 1981; Heikkila and Schultz, 1984; Welch, 1985; Anathan *et.al*, 1986; Munro and Pelham, 1986; Pelham, 1986; Subjeck and Shyy, 1986; Anson *et.al*, 1978; Hoffman *et.al*, 1987; Simon *et.al*, 1987)

cultures (Burdon, 1986; Subjeck and Shyy, 1986). Also, the induction of an oxidative state is also correlated with the accumulation of phosphorylated adenylated dinucleotides (e.g. AppppN) generated by tRNA synthetase (Lee *et.al*, 1983).

The term phenocopies was introduced by Goldschmidt in the 1940s to describe environmentally induced developmental defects which resemble anatomically, genetically determined phenotypes. It has been suggested that phenocopies are the result of environmental alteration of the developmental program of transcription and translation during critical periods of organogenesis. Disruption of this embryonic program leads to the production of anatomic malformations (Subjeck and Shyy, 1986; Petersen and Mitchell, 1987). Hyperthermia which indirectly influences alteration of gene expression has been shown to cause congenital phenocopies such as microphthalmia, microencephaly and anencephaly in a variety of animals (Mirkes, 1987). The induction by hyperthermia of *Drosophila* multiple wing hair phenotype and segmental anomalies in the chick embryo has been suggested to be a molecular effect due to phenocopy induction resulting from the non-coordinate recovery in the synthesis of specific gene products required at that stage in the developmental sequence (Primmett *et.al*, 1988). Furthermore, temperature elevation has also been attributed to the delay in cell cycle progression, the precocious appearance of the grey crescent (initiation site of gastration) in axolotl eggs and morphological damage of the *Xenopus laevis* larvae epidermis (Beetschen, 1987; Petersen and Mitchell, 1987; Mizzen and Welch, 1988).

Heat Shock Proteins

The increased sensitivity and resolution of two-dimensional gel electrophoresis has helped to elucidate the number of eukaryotic HSPs to be

greater than fifty. Most are characteristically acidic (pI-5.0-6.5). In addition, at least seventeen prokaryotic HSPs have been observed. These new polypeptides each with its own intracellular localization are designated according to their molecular weights, estimated from SDS-polyacrylamide gel electrophoresis. The major HSPs of most organisms fall into three classes (Table III): the small eukaryotic HSPs (15-30kDa), the HSP70-like (68-78kDa), and the HSP83-like (80-110kDa) polypeptides (Alahiotis, 1983; Neidhardt *et.al*, 1984; Burdon, 1986; Vierling *et.al*, 1988).

A problem when evaluating the HSP spectrum of an organism by two-dimensional electrophoresis has been the complexity due to the presence of isoform variants located within each major HSP class. The relative number and amounts of the isoforms seem to vary in respect to cell type and the stress agent used to elicit the response (Welch and Feramisco, 1984). Genetic and biochemical evidence, indicates that these isoform variants are highly related. One interpretation invokes post-translational modification of the parent polypeptides (see Table III). Such modifications may affect both the function and metabolic stability of HSPs. It is known that their metabolic stability can vary and can be influenced by nutritional status (Lanks, 1983). Results by Welch (1985) indicate that some reagents such as fresh serum, phorbol esters or calcium ionophores can cause an increase in isoforms (via phosphorylation) without an increase in HSP synthesis. Alternatively, the existence of multigene families for particular HSPs has been observed and thus a number of the protein variants could be products of the closely related genes (Ashburner and Bonner, 1979; Neidhardt *et.al*, 1984; Welch, 1985; Burdon, 1986; Barnier *et.al*, 1987).

Table III. Three Major Eukaryotic HSP Classes

Class	Size range (kDa)	Species	Covalent Modifications	Intracellular Location/Comments
HSP83-Like	110	Mammals		Nucleolus
	92-95	Vertebrates	Phosphorylation	Golgi
	95	Plants	Phosphorylation	
	83-90	Vertebrates	Methylation, phosphorylation and ADP-ribosylation	Cytosol
	84	Yeast		
	84	*Drosophila*		
	80	Plants		
HSP70-Like	68-78	All	Phosphorylation not all species Methylation	Cytoplasmic, migration to nuclues and nucleolus after stress. Basal HSP levels in cytosol and organelle in unstressed cells. *E. Coli* equivalent is B66.0 (dnaK protein)
Small HSPs	23-30	Vertebrates	Glycosylation	
	22	*Drosophila*		
	23			
	26			Nuclear matrix after stress.
	28			
	26	Yeast		
	15-27	Plants		Cytosol and chloroplasts.

(Burdon, 1986; Lindquist and Craig, 1988)

HSP70-Like Class

The most predominant and highly conserved HSPs in all species, with the exception of plants, are the HSP70-like proteins. Plants contain greater levels of the smaller HSPs relative to the HSP70-like proteins (Vierling *et.al*, 1988). The focus of this HSP literature review shall emphasize HSP70-like proteins because of their prominence, evolutionary conservation, inducibility and the developmental regulation of expression and their putative functions. Note, the nomenclature 'HSP70' shall be used when the identity of the heat shock 70kDa-like protein(s) cannot be unambiguously classified as inducible (hsp) or cognate (hsc).

Intrinsic ATP-binding properties of the HSP70-like class have enabled Welch and Feramisco (1984) to develop a rapid isolation technique based on DE52 ion-exchange chromatography followed by affinity chromatography on ATP-agarose. This technique coupled with previous studies has helped characterize and further sub-divide the eukaryotic HSP70 multifamily. Three main HSP70-like proteins: hsp70 (stress inducible), hsc70 (for heat shock cognate; constitutively expressed) and grp78 (glucose regulated protein) each with distinct intracellular locations and regulatory patterns of expression (Table IV) have been isolated from mammals'. An additional member of this family (hsx70) that is closely related to hsp70 has recently been found in primate cells (Pelham, 1986; Dworniczak and Mirault, 1987). Craig *et.al* (1987) postulated that the multiple forms of HSP70 proteins can complement, at least partially, for each others individual function(s). Under these circumstances, the normal expression pattern of some of the HSP70 genes must change in order for intergenic complementation to occur.

A semantic problem of the highly-related multifamily of HSP70-like polypeptides arises due to species specific nomenclature of these stress

Table IV. Eukaryotic HSP70-Like Family

Class	Sub-division	Regulation	Intracellular location
HSP70-Like	hsp	Highly inducible; devel. regulated	Basal nucleus and cytosol; migration to nucleus, nucleolus and cytoplasm after stress induction.
	hsx (Primates)	Stress inducible but high basal level; cell cycle regulated; E1A induced	Basal nucleus and cytosol; migration to nucleus, nucleolus and cytoplasm after stress induction
	hsc	Constitutive expression; high in growing cells and lower in resting; slightly heat inducible	Basal levels in cytosol filament association; migration to nuclear region and cytoplasm
	grp	Basal levels but can be induced by glucose de-prevention(underglycosylation) and malfolded proteins; slight heat induction	Lumen of ER restricted

(Pelham, 1986)

proteins. Rodent hsp70 and hsc70 are often called hsp68 and hsp70 while murine hsc74 is probably grp78 (Burdon, 1986). Human hsx70, hsc70 and grp78 have been referred to as 72K, 73K and 80K, respectively (Burdon, 1986). Hsx70 is frequently called hsp70 and not distinguished from this protein (Pelham, 1986; Subjeck and Shyy, 1986). Unification of HSP70-like nomenclature based on a number of variables and intrinsic protein properties (i.e. molecular weight, induction and regulation, post-translational modifications and sequence homologies) will alleviate some ambiguity between inter/intraspecie comparison of HSP70-like protein characteristics.

An exception to the HSP70-like multifamily of eukaryotes has been observed in prokaryotes which contain a single hsp70 protein designated B66.0, the product of the dnaK gene. The purified dnaK protein also has a very weak ATPase activity and is capable of autophosphorylation of its threonine residue (Lindquist, 1984; Neidhardt *et.al*, 1984).

Mitchell *et.al.* (1985) have revealed in studies on HSP70-like proteins, the problem of degradation *in vitro*. This occurs even during electrophoresis and does not appear to be mediated by general proteinase, but rather the HSP70s have a slow autoproteolytic action based on the structural potential of each.

Evolution and Conservation of HSPs

The heat shock response in which cells respond to stress stimulus by producing a highly conserved set of HSPs, appears to be ubiquitous throughout nature. Approximately 1.8×10^9 years have passed since the ancestors of human and *Escherichia coli* (*E. coli*) diverged (Ayala, 1976) and only a very small number of chloroplast and mitochondrial proteins are known to be as evolutionarily conserved as the heat shock proteins (Lindquist, 1984; Bardwell and Craig, 1987; Rebbe *et.al*, 1987).

Genetic and immunological cross-reactivity analyses have revealed that the most highly conserved HSPs is the HSP70-like and to a lesser extent, the HSP83-like proteins (Moran, 1982). The derived human inducible hsp70 amino acid and nucleotide sequence has an average homology of 76.5% to the *Drosophila* hsp70, 79.5% to hsc70 (the presumed product of the *Drosophila* cognate gene), 74.5% to yeast hsp70 (SSA1 gene) and 55.5% homologous to the dnaK protein (hsp70) of *E. coli* (Voellmy *et.al*, 1985; Chappel *et.al*, 1986; Mues *et.al*, 1986). Recently, human grp78 has been shown to be structurally related to hsp70 and to be identical to the immunoglobulin heavy chain binding protein "BIP" in B lymphoid cells (Dworniczak and Mirault, 1987).

Bardwell and Craig (1987) isolated and sequenced the *E. coli* gene htpG encoding the heat shock protein, C62.5. The predicted amino acid sequence of C62.5 was found to be 41% and 42% identical to the *Drosophila* and human hsp83 proteins, respectively. Some regions of hsp83 are greater than 90% conserved in all species for which the sequence is known. Extensive homology at both the nucleotide and amino acid level of the human hsp90 protein correlated well with that determined for both the *Drosophila* hsp83 (78%) and yeast hsp90 (61%) (Bardwell and Craig, 1987). Hydrophobicity/hydrophilicity indices plots of HSPs located within the two classes reveal additional highly conserved properties (Rebbe *et.al.*, 1987).

The smaller HSPs (15-30kDa) are not so well conserved in phylogenetic terms as no prokaryotic homologs have been identified by heterologous hybridization. This indicates that the small HSP genes are probably derived from a common nuclear eukaryotic ancestor (Vierling *et.al*, 1988). Nucleic acid sequence analyses have demonstrated some homology amongst the small hsps of insects, vertebrates, plants and nematodes

(Burdon, 1986). The four small HSPs of *Drosophila* have 40% similarity in amino acid sequence to the mammalian α-crystallin proteins (20-30kDa), a major structural component of vertebrate eye lens (Ingolia and Craig, 1982a).

Genetically engineered chimeric genes containing the inducible hsp70 promoter of *Drosophila* have been used to produce a number of transgenic eukaryotic organisms (Voellmy *et.al*, 1985; Rancourt *et.al*,1987; Spena, 1987). Induction in response to elevated temperature of the heat inducible chimeric genes in transgenic organisms implies a common and evolutionarily conserved mechanism(s) of transcriptional regulation (Bond and Schlesinger, 1986; Finley *et.al*, 1987; Wiederrecht *et.al*, 1987).

The extensive conservatism between diverse organisms suggest that larger HSPs have a common evolutionary ancestor and perform functions that are of fundamental importance to all cells. Furthermore, the high homology of common domains encoded by the multigene family of mammalian HSP70-like polypeptides may be indicative of the HSP70s' functionally distinct and overlapping plasticity (Plesofsky-Vig, 1985; Pelham, 1986).

Genetic Organization of the Heat Shock Genes

The genetic organization of the heat shock genes have been extensively studied in the *Drosophila*. The three major HSP classes have been mapped, cloned and sequenced (Mckenzie *et.al*, 1975; Livak *et.al*, 1978; Schedl *et.al*, 1978; Corces *et.al*, 1980; Wadsworth *et.al*, 1980).

Early results from *in situ* hybridization experiments and determination of the electrophoretic mobility of recombinant restriction digestion were consistent with the localization of four small heat shock genes encoding the proteins hsp27, hsp26, hsp23 and hsp22 to a fifteen kilobase

(Kb) DNA region. This region is contained within a single inducible puff site at locus 67B on the left arm of chromosome three (Voellmy *et.al*, 1981). Recently, three other genes, (1, 2 and 3) have been discovered though their putative proteins have not been identified (Pauli *et.al*, 1988). Evidence for seven genes at one puff site indicated an oversimplification of a proposed model in which each puff locus was speculated to contain a single gene for one HSP product (Ingolia and Craig, 1982a).

The absence, as visualized by electron microscopy, of any DNA loops within the heteroduplex hybrids and the appearance of a single S1-resistant DNA:RNA band indicated a lack of intervening sequences contained in these inducible small heat shock genes (Wadsworth *et.al*, 1980). The absence of introns within the heat shock genes has been shown to be common only to those genes that are inducible (hsp) and not to the developmentally regulated cognate heat shock (hsc) genes (Corces *et.al*, 1980).

Sequence analysis of these small contiguous hsps genes by Ingolia and Craig (1982a) revealed partial homology among themselves and may thus be a result of duplications from an ancestral gene. Furthermore, three of the hsp genes at this locus exhibited alternating polarities and thus are not transcribed in the same direction. Northern analysis has revealed a lack of any large precursor sense/antisense transcripts, thus indicating individual gene promoter transcription, though a common promoter can not be ruled out. Local events such as regional unfolding of the chromosome at this locus renders these tandemly linked hsp genes simultaneously available for coordinate expression induced by stress agents (Ashburner and Bonner, 1979; Voellmy *et.al*, 1981). In addition, these small hsp genes have been found to be independently expressed at several stages of development (Pauli *et.al*, 1988).

In situ hybridization of *Drosophila* salivary glands using tritiated hsp70 RNA, isolated from sucrose gradients, revealed hybridization to its complementary chromosomal DNA at sub-division 87B (Mckenzie *et.al.*, 1975). Sensitive cytological studies using cloned and cDNA hsp70 genes has identified the exact location of six hsp70 copies on the right arm of chromosome three; two at locus 87A7 and four at locus 87C1 (Schedl *et.al*, 1978; Craig *et.al*, 1979; Holmgren *et.al* 1979; Mirault *et.al*, 1979). Each of the hsp70 genes is organized within a 2.5Kb conserved element consisting of a 2.1 Kb mRNA coding region and a 0.4Kb 5' region which is not transcribed (Moron *et.al*, 1979; Artavanis-Tsakonas, 1979). The hsp70 genes at locus 87A7 are approximately 1.7Kb apart and are arranged as two inverted repeats in opposite orientation that are transcribed from opposite strands (Mirault *et.al*, 1979). Three of the hsp70 gene copies at 87C1 are tandemly arranged and separated by a large insert (38Kb) from the fourth copy oriented in the opposite direction (Moren *et.al*, 1983). The intervening stretch of DNA contains more than twenty copies of the heat inducible α-ß repetitive units. The function of the α-ß sequence remains unclear (Ish-Horowicz and Pinchin, 1980).

The apparent fluidity of the genome at the loci 87A7 and 87C1 may be due in part to inter/intramolecular recombination events promoted by hsp70 gene duplication. There is strong circumstantial evidence that one class of these events, gene conversion, are relatively frequent. Gene conversion is a meiotic process of directed change in which one allele directs the conversion of a partner allele to its own form. Other rearrangements may be catalyzed by insertion elements which are known to be present at both loci (Moran *et.al*, 1983) or the process of retroposition in which RNA transcripts are copied into DNA and integrated into new genomic sites

(Deininger and Daniels, 1986). An autonomous gene, hsp68 located at cytological locus 95D, has been shown to related to the inducible hsp70 multigene family. No intervening sequences have been found in the genes encoding for either hsp70s or hsp68 (Ashburner and Bonner, 1979, Craig *et.al*, 1983).

The developmental and constitutively regulated hsc70 genes (Hsc1-Hsc7) have been localized to interdispersed sites (loci 70C, 87D 10E, 88E, 50E and 5C, respectively) that are not puff inducible (Craig *et.al*, 1983; Lindquist and Craig, 1988). Hsc1 and Hsc2 have been found to contain an intervening sequence similar to that of the *Drosophila* hsp83. This protein, hsp83, is unique in that it is expressed at both ambient and slightly elevated temperatures even though it contains an intron (Corces *et.al*, 1980; Ingolia and Craig, 1982b; Craig *et.al*, 1983; Palter *et.al*, 1986). Yost and Lindquist (1986) have demonstrated that the splicing of mRNA precursors is blocked in *Drosophila* cells at the upper temperature range of the heat shock response. Thus heat shock genes that are free of introns by-pass the block in RNA processing at extreme temperatures and are translated into functional stress proteins. Recently, three additional *Drosophila* genes homologous to hsp70 have been isolated, bringing to thirteen the number of isolated HSP70 family members in *Drosophila* (Craig *et.al*, 1987).

If as an organism becomes more complex it requires more genetic diversity to regulate its development, one would expect simple organisms to contain fewer multicopies of gene families (Ayala, 1976). This statement is valid for the HSPs as all organisms studied to date contain a multifamily of HSP70-like genes with the exception of the prokaryotes. *E. coli* contains only one heat inducible hsp70 gene (dnaK) (Neidhardt *et.al*, 1984). Eukaryotes such as yeast, *Saccharomyces cerevisia*, contain at least nine copies related

to the HSP70-like genes. Eight of these genes originally named YG100-YG107, have been renamed on the basis of functional and structural similarities: SSA1-4 on the left arm of chromosome 1 (stress seventy family A; YG100, YG102, YG106, YG107, respectively); SSB1 and SSB2 (YG101 and YG103, respectively); SSC1 on the right arm of chromosome X (YG104); and SSD1 (YG105). Recently another member of this family, the KAR2 gene, has been identified on the left arm of chromosome X (Craig *et.al*, 1987; Slater and Craig, 1987; Lindquist and Craig, 1988).

Higher eukaryotes (e.g. humans) have at least ten different genes or pseudogenes located on chromosomes 6, 14, 21, and on at least one other chromosome (Lindquist and Craig, 1988). Some of the human cognate hsc70 genes contain as many as eight introns (Dworniczak and Mirault, 1987). All eight genomic hsp70 clones of the human were found to exhibit extensive flanking regions of 'Alu' repetitive DNA. The 'Alu' regions, descended from the 7SL RNA gene, may play a role in genomic rearrangements via translocational changes (Deininger and Daniels, 1986; Mues *et.al*, 1986).

The simplest explanation for multiplicity of hsp70 genes is that it confers a selective advantage permitting more rapid accumulation of heat shock proteins after stress (Moran *et.al*, 1983).

The Mechanism of Eukaryotic HSP Induction

It has been noted that the stress response is triggered by a variety of environmental and biological agents (see Table II). Since the response to these perturbations is extremely rapid, many scientists believe that the primary control of the heat shock response is at the transcriptional level. It was proposed that some cellular target, upon which all of the stimuli might act, generates a common signal that would activate HSP transcription

(Ashburner and Bonner, 1979; Neidhardt *et.al*, 1984; Burdon, 1986; Lindquist and Craig, 1988).

The heat shock response is such a complex phenomenon that a simple one step induction mechanism would seem unlikely. A great number of the inducing agents listed in Table II seem to be targets for uncouplers of oxidation phosphorylation and inhibitors of the electron transport. Sin (1975) studying heat shock and non-heat shock supernatants of isolated *Drosophila* salivary mitochondria, postulated that the intracellular signal(s) that produced the heat shock response were mitochondrial in origin. More recent evidence suggests however, that mitochondria are unable to play a direct role in heat shock gene expression. The contribution of an oxidative stress state via changes in mitochondrial intracellular membrane permeabilities and disruption of the electron transport may indirectly affect HSP transcription however.

Strong support for a common and evolutionarily conserved intracellular transcriptional signal was prevalent when hsp70 genes from *Drosophila* were introduced into mouse cells, monkey cells, sea urchin embryos, *Xenopus* oocytes or yeast. The exogenous hsp70 genes were actively transcribed only when the recipient cells were subject to hyperthermia. This signal was referred to as the heat shock transcriptional factor (HSTF) (Corces *et.al*, 1981; Pelham, 1982, 1985; Bienz, 1984; Voellony and Rungger, 1982).

Structural analysis of heat shock genes isolated from several eukaryotic organisms, including plants, has revealed homologous DNA promoter regions. Ingolia *et.al* (1980) compared 5' flanking sequences of three distinct hsp70 genes and observed the putative promoter sequence TATAAATA (TATA box) 325bp upstream from the presumptive protein

initiation codon (ATG). The heptanucleotide 'cap' site was located 30bp downstream from the TATA box while a 12bp sequence with dyad symmetry, 5'C--GAA--TTC--G3', begins 23bp upstream from the beginning of the above A-T rich sequence. Serfling *et.al* (1985) stated that he upstream sequences of protein-coding genes may function in: (1) the general stimulation of transcription; (2) tissue-specific gene expression; and/or (3) the induction (or the repression) of transcription by the action of specific agents.

RNA analysis of 5' deletion mutants of the *Drosophila* hsp70 gene (Pelham, 1982; Dudler and Travers, 1984) revealed that the promoter region between -10 bp to -66 bp was sufficient for heat induced promotion of mRNA transcription. Regions within the 5' promoter for hsp83 and hsp70 when mapped for Exo III resistance (protein-binding DNA; "footprinting") was found to contain two sites resistant to Exo III digestion during heat treatment (Wu, 1984; Drabent *et.al*, 1986). Within this region was found the TATA box sequence which directs RNA polymerase II binding and the upstream hyphenated dyad consensus sequence now referred to as the heat shock element (HSE) (Pelham, 1982). It was postulated by Parker and Topol (1984a) that the TATA box and the imperfect inverted repeat HSE are two independent protein-binding cis-regulators of heat shock gene transcription. This stress induced promoter complex causes a change in chromatin configuration. The configurational change stimulates RNA polymerase II affinity and hence efficiency, for hsp mRNA transcriptional production.

To date it is known that the promoter DNA sequences (i.e. HSE) involved in the heat shock response have been strongly conserved during evolution. All eukaryotic organisms contain multiple copies of tandemly reiterated HSEs of varying proximity to the TATA box. The HSE is necessary

and sufficient to confer heat inducibility upon an adjacent TATA box and is the binding site for an activated HSTF. Efficiency of HSP transcription is positively correlated to the proximity of the HSE relative to the TATA box, indicating a chromosomal position effect. Furthermore, the additive effects of the HSE copies, which are cooperatively bound, also contribute to the efficiency of HSP expression (Topol *et.al*, 1985; Baumann *et.al*, 1987; Hoffman *et.al*, 1987; Maniatis *et.al*, 1987).

Slater and Craig (1987) studying yeast hsp70 transcriptional regulation stated that though HSE seems to be a positive cis-regulating element; a role for negative regulation in modulating heat shock expression involving sequences flanking the HSE adjacent to the TATA box cannot be ruled out. It is plausible that some eukaryotic HSEs with basal constitutive activity may be regulated by antagonistic negative elements (which are derepressed upon heat shock) in their heat shock promoters to provide a transient burst of heat shock transcription.

Recently, Wu *et.al* (1986, 1987) detected, by 5' hsp70 promoter deletion, a second regulatory domain distinct from the one that is required for heat shock (HSE) and metal induction. This second regulatory domain, upstream of the HSEs, requires serum for induction of the hsp70 genes. The basal serum-regulated promoter was shown to contain three protein-binding elements, an inverted "CCAAT" (CAT-motif), a serum-regulated element (SRE) and a TATA box. The "CCAAT" is referred to as an upstream promoter element and it confers an increased rate of transcription (Maniatis *et.al*, 1987). Dworniczak and Mirault (1987) verified that the human hsc70 gene also contained a CAT-motif and a functional HSE that caused a two-fold mRNA accumulation upon hyperthermia.

In 1984a, Parker and Topol isolated the evolutionarily conserved eukaryotic HSTF and a chromatographically distinct B factor from *Drosophila* K_c cell nuclear extracts. The B factor and a previously isolated A factor are necessary *Drosophila* RNA polymerase II transcription factors that bind to a region of DNA that includes the TATA box. HSTF, A and B factors are present in both heat shocked and non-shocked K_c cells. Interestingly, HSTF and A factor affinity and transcriptional efficiency in heat shocked cells is increased while the B factor is inversely affected. Parker and Topol (1984b) concluded that the *Drosophila* hsp70 gene expression can be induced to express at high levels by the binding of the A factor and the activated HSTF. The B factor contribution was negligible during stress induction of hsp70 though it may play a role in basal level expression or negative regulation of hsp70 transcription under physiological conditions.

Cells incubated with protein synthesis inhibitors (i.e. cycloheximide) still retain a functionally active HSTF upon heat shock. These results suggest that HSTF exists in a non-binding inactive state or a basal transcriptionally binding form which is converted upon appropriate stimulus to an active, high affinity transcriptional regulator (Kingston *et.al*, 1987; Zimarino and Wu, 1987). Sorger *et.al* (1987) have found that the conversion of the inactive HSTF to an active form is mediated by heat induced multiple phosphorylation via post-translational modification in yeast. Thus it has been proposed that the degree of reversible post-translational modification of a pre-existing HSTF results in either an increase in HSE-binding activity and/or an increased efficiency of bound HSTF with other components of the transcriptional machinery (Sorger and Pelham, 1988).

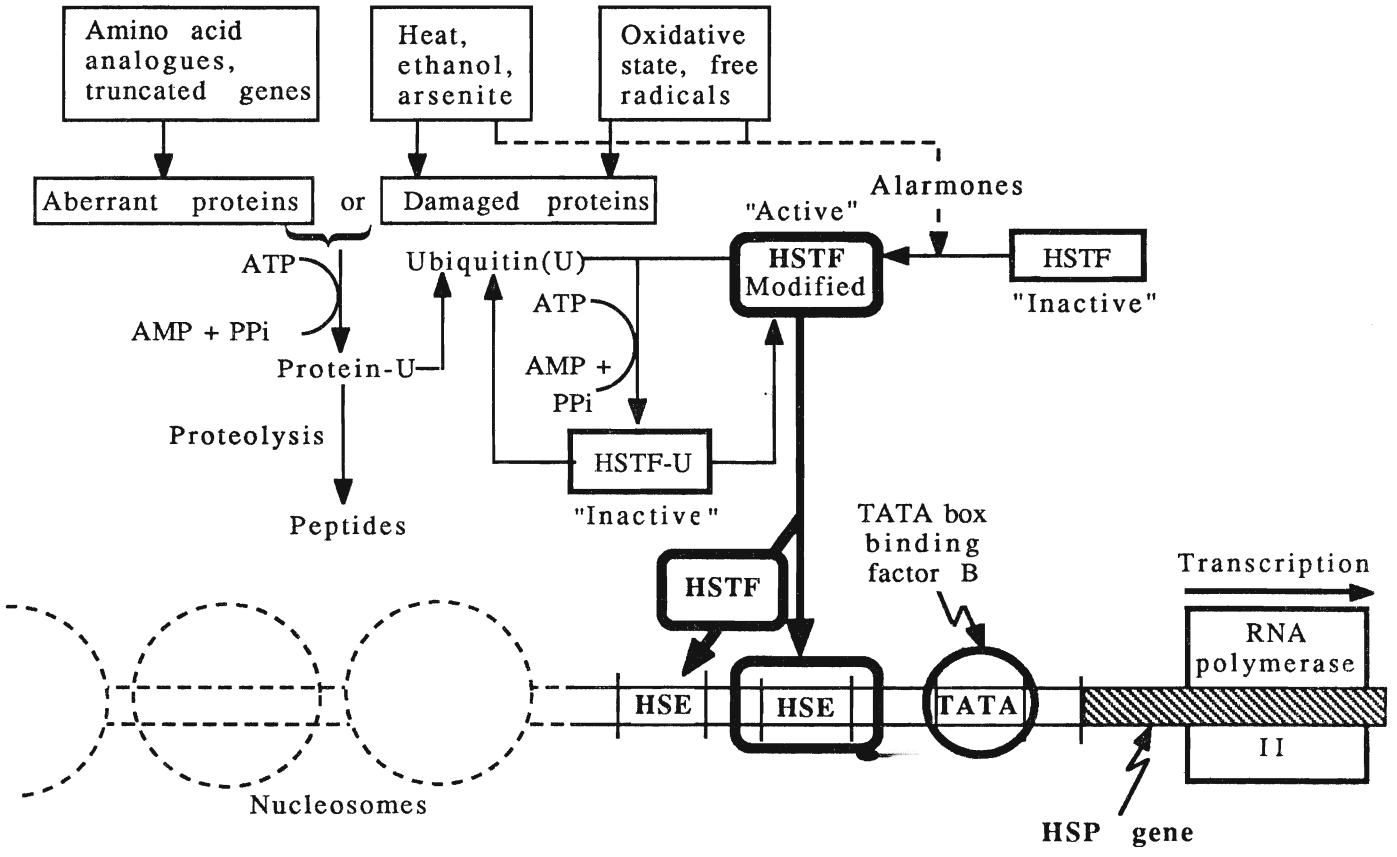
Sequence specific DNA-affinity chromatography using HSE sequences has led to the purification to homogeneity of HSTF from *Saccharomyces,*

Drosophila, human and HeLa cells (Kingston *et.al*, 1987; Sorger and Pelham, 1987; Sorger *et.al*, 1987; Wiederrecht *et.al*, 1987; Zimarino and Wu, 1987). This cross-reactive HSTF has been found to have variable sizes ranging from 110kDa in *Drosophila* to 150kDa in yeast. All isolated HSTF contain a protease resistant 70kDa DNA-binding fragment. The discrepancy in molecular weights for HSTF is thought to represent an inherent artefact of electrophoretic mobility of the HSTF-HSE complex in a non-denaturable structure and/or the induced multiple covalent modifications of HSTF. Recently, Wiederrecht *et.al* (1988) isolated from a λgt11 expression library the yeast HSTF gene (HSF1) encoding for a 833 amino acid protein of 93.22kDa. The DNA-binding domain was localized to a 118 amino acid region in the N-terminal third of the protein. This DNA-binding region showed no resemblance to any secondary structural motifs such as the zinc finger (Dressler and Gruss, 1988) or the α-helical leucine zipper (Landschulz *et.al*, 1988) which characterize many eukaryotic transcription activators (Dyan and Tjian, 1985). Thus one observes that the differential activation of the heat shock mRNA by stress agents or developmentally regulated signals is a multifaceted combination of complexed interactions involving modified and unmodified DNA-binding proteins and their respective DNA domains.

The nature of this HSTF conversion to a highly transcriptionally active state relates to the heat shock induction mechanism. As previously described (Table II), many stress agents induce aberrant proteins and oxidative states. It has been proposed by Lee *et.al* (1983) and Findley *et.al* (1984) that the stress induced accumulation of abnormal and damaged proteins and/or phosphorylated adenylated dinucleotides acting as "alarmones" (i.e. regulatory molecules) could cause the indirect activation of the pre-existing HSTF (Figure III). The production of aberrant protein and its effect on HSTF

Figure III. A schematic representation of the mechanism for heat shock gene activation. Stress agents may induce aberrant proteins that are recognized by the ATP-dependent ubiquitin degradation system. The competition and ultimate depletion of ubiquitin by damaged proteins liberates pre-existing HSTFs from its inactive ubiquitinated state. The mechanism of HSTF activation may also involve structural alterations, of HSTF, induced by oxidative states. Active HSTFs bound to the upstream HSEs of the HSP promoter, in the presence of TATA box binding factor A, allows for the transcription of the HSP gene by RNA polymerase II.

Figure III. Putatuive HSTF Activation for HSP Transcription



(Burdon, 1986)

activation has gained support by the observation that transgenic *Xenopus laevis* oocytes containing the assayable heat inducible hsp70-ß-galactosidase hybrid gene was expressed only after microinjection of denatured proteins (Ananthan *et.al*, 1986).

Under normal physiological circumstances, aberrant proteins are bound via peptide bonding to the N-terminus of a highly evolutionarily conserved 76 amino acid protein termed ubiquitin (Parag *et.al*, 1987). Ubiquitin, an ATP-dependent proteolytic system in eukaryotes, is a developmentally regulated multigene family containing multicopies of tandemly arranged protein-coding sequences that encode for a polyubiquitin 1.7Kb-3.5Kb precursor mRNA (Bond and Schlesinger, 1986; Findley *et.al*, 1987; Ovsenek and Heikkila, 1988). Once conjugated to a substrate, ubiquitin usually targets the degradation of the protein into amino acid residues via liposomal protease, though exceptions such as the non-degraded ubiquinated histone 2A complex exists (Glover, 1982).

It was the above mentioned exception that lead Munro and Pelham (1985) to speculate that the inactive HSTF is ubiquinated in a non-degradable form but with the ubiquinated moiety being continually removed and replaced. Stress agents that cause a fall in the cellular ubiquitin pools by the production and accumulation of aberrant protein substrates, might result in an increased level of HSTF in a non-ubiquitinated form that may be activated by covalent modification (see Figure III). According to this model, the rate-limiting step in the deactivation of hsp genes is the degradation of large quantities of abnormal proteins. Thus one would expect ubiquitin synthesis to increase during and after periods of induced hsp gene expression (Burdon,1986). This prediction has been found to be valid by Bond and Schlesinger (1986) and Findley *et.al* (1987). They determined

that the DNA sequences of chicken ubiquitin (Ub1) and yeast polyubiquitin genes both contain an upstream heat shock consensus element (HSE). The expression of the ubiquitin gene(s) exhibits a transient yet coordinate heat inducible pattern comparable to that of HSP induced expression and developmental stage-dependent hsp70 and hsp87 expression in *Xenopus laevis* tadpoles (Krane and Heikkila, 1988; Ovsenek and Heikkila, 1988).

Evidence of a second oxidative induced alarmone pathway (via AppppN) for HSTF activation is supported by use of a temperature sensitive ubiquitin mutant mouse cell line (ts85). It was observed that no substrate conjugation by ubiquitin is possible at non-permissive temperatures, yet hsps are still synthesized (Findley *et.al*, 1984). The interpretation of this result is ambiguous as the conclusion could support either; 1) a putative second alarmone pathway to HSTF activation via post-translational modification (i.e. phosphorylation) through some unknown mechanism or 2) the ubiquitinated-HSTF model (Findley *et.al*, 1984) depending upon the affinity of this conjugated complex or 3) a non-conjugated ubiquitin/HSTF competitive model of Ananthan *et.al* (1986). Thus, the mechanism of induction generating an active post-translationally modified HSTF from a pre-existing HSTF is still unclear though a specialized role for ubiquitin and adenylated dinucleotide alarmones during heat shock can be inferred.

The Mechanism Bacterial HSP Induction

The prokaryotic regulation of the heat shock response appears to operate mainly at the level of transcription. The HSP synthesis is brought about by the stimulation of gene transcription by an active regulatory system and not by the direct effect of heat on the corresponding genes (Fujita and Ishihama, 1987a).

A single effector of the bacterial heat shock response was discovered in temperature sensitive suppressor mutations. Originally called HtpR, a product of the htpR gene, this gene bears the alternative designation rpoH. The effector molecule was identified on gels as protein F33.4 though it is now referred to as sigma^{32} (σ^{32}), (Burdon, 1986; Straus *et.al*, 1987). The gene was found to contain two major promoters, of which the downstream P2 promoter was enhanced transiently upon heat shock, while the upstream P1 promoter was not. This finding supports the concept that each of the multiple promoters within single genes or operons has a different regulatory role (Fujita and Ishihama, 1987a).

The mechanism of induction for the rpoH P2, Eσ^{70}-dependent transcription promoter is still unclear though similarities to the eukaryotic inductive model of aberrant protein production and phosphorylated adenylated dinucleotides have been implicated (see Figure III). One attractive feature of the adenylated dinucleotide model is that different stresses produce different patterns of nucleotide pools, and this offers the possibility of accounting for the differences in the synthesis of individual proteins in response to diverse stimuli. Furthermore, one of the bacterial heat shock genes encoding the major intracellular protease, protease La, parallels the finding of ubiquitin being a eukaryotic heat shock protein (Neidhardt *et.al*, 1984; Munro and Pelham, 1985; Ananthan *et.al*, 1986; Privalle and Fridovich, 1987; Straus *et.al*, 1987).

The product of the rpoH gene, σ^{32}, encodes for a protein of 32kDa having comparable homology to the C-terminal half of RNA polymerase sigma factor (σ^{70}). The σ^{32} protein interacts with the core form of RNA polymerase to form the holoenzyme (Eσ^{32}) having analogous function to the major transcriptional regulating holoenzyme Eσ^{70} though 45% smaller in size

(Neidhardt *et.al*,1984; Fujita and Ishihama, 1987b). The holoenzyme $E\sigma^{32}$ has an absolutely distinct promoter selectivity from that of $E\sigma^{70}$. $E\sigma^{32}$ is a positive regulator for the recognition and initiation of transcription for the heat shock promoter genes such as groE, dnaK, rpoD and CC62.5, but cannot recognize the promoters which are usually transcribed by $E\sigma^{70}$ (Fujita *et.al*, 1987a, 1987b). *In vitro* studies have shown that bacterial RNA polymerase can transcribe the major *Drosophila* heat shock genes, indicating the diverse conservation of the hsp induction mechanism (Alahiotis, 1983).

Finally, adding further complexity to the eukaryotic and prokaryotic (Neidhardt *et.al*, 1984) heat shock gene regulation, there is evidence that the transcriptional and post-transcriptional control of the heat shock genes is autoregulatory, i.e. the concentration of induced hsps determine the activity of the heat shock genes. DiDomenico *et.al* (1982a) established that *Drosophila* heat shock mRNAs remained stable at 25^0C if the synthesis of hsps was blocked by cycloheximide. They suggested that the hsp70 protein accumulation regulates hsp gene deactivation and hsp mRNA destabilization in the eukaryotes while the dnaK gene product (hsp70) regulates the rpoH gene at the transcriptional level.

Stress Induction of Transcriptional and Translational Controls

It has been well established in *Drosophila* that certain stress agents cause transient and reversible reduction in synthesis of the normal cellular proteins while at the same time inducing preferential synthesis of the hsps. This implies that non-heat shock genes and heat shock genes are controlled at the level of transcription and translation. The degree of conservation of species specific transcriptional and translational controls are unknown during stress induction (Alahiotis, 1983; Burdon, 1986).

Unlike most species studied to date, where the individual synthesis of heat shock proteins is an indirect result of *de novo* transcription of hsp genes, in *Xenopus laevis* oocytes, hsp70 induction is controlled exclusively at the translational level (Burdon, 1986). Heat induced expression of hsp70 in enucleated or α-amanitin treated *Xenopus* oocytes led Bienz and Gurdon (1982) to predict that the hsp70's mRNA pre-exists in the cytoplasm of non-heat shocked oocytes in a masked form. Direct evidence for the pre-existing hsp70 mRNA was obtained only after polysomal RNA was isolated and translated *in vitro* from non-heat shocked oocytes. *In vitro* translation of extracted mRNA (non-polysomal) from non-induced oocytes failed to produce the endogenous hsp70 protein (Bienz and Gurdon, 1982). Two-dimensional electrophoresis of *in vitro* translation products from non-heat shocked human thymus cells adds further support to the existence of translational control of pre-existing hsp70 mRNAs (Colbert and Young, 1987).

The exact mechanism for mRNA masking of pre-existing tanscripts is unclear. Possibilities may involve: (1) a temperature sensitive binding of proteins or complementary antisense RNA to exon/intron domains within the mRNA which may interfere with their translation; (2) a poly(A)$^+$ protein-binding complex (a putative HSP); (3) compartmentalization of the hsp mRNA or (4) containment of mRNA in mRNP particles (Bienz and Gurdon, 1982; Tabak and Grivell, 1986).

Attention has recently been focused on the reduction or inhibition of non-hsp gene transcription during the stress response. Hypermethylation of DNA cytosine residues (5-methylcytosine) and covalent modifications of core histones may cause inactivation of gene transcription by interfering with cis-regulatory DNA-protein interactions and chromatin conformation, respectively (Cedar, 1988; Hebbes *et.al*, 1988). Interestingly, methylation

interference studies artificially produced in the human 5' hsp70 promoter, inhibited binding of the active HSTF. Nucleotide analysis revealed that methylation within the HSE of three residues on the sense strand and two on the antisense strand inhibited HSTF binding and thus transcription (Kingston et.al., 1987).

Thin layer chromatography and electophoresis of renatured histone bands H2B, H3 and H4 isolated from SDS-PAGE gels have demonstrated that heat shock or arsenite alters the pattern of histone methylation in *Drosophila* cells. Heat shock induces an increase in proline methylation in H2B concurrently with a decrease in lysine methylation and the appearance of methylated arginines in histone H3. The N-terminus of H2B has been implicated with linker DNA interaction, thus changes in methylation of H2B by stress could cause restructuration and stabilization of chromatin. This could lead to inhibition of transcription and protection of the transcriptional products from the cellular increase in proteolytic activity (Desrosiers and Tanguay, 1988). Accompanying this heat induced histone modification, is a non-coordinate increased expression of H2B and reduced synthesis of the other core histones (Tanguay *et.al*, 1983).

Results from studies in *Xenopus* oocytes fit the pattern of previous work in *Drosophila* (Burdon, 1986) that hyperthermia strongly disturbs and stabilizes 40S ribosomal RNA processing and production, respectively. The 40S precursor contains the coding regions for the mature 5.8S, 18S and 28S RNA ribosomes. Inhibition of hsp synthesis in *Xenopus* oocytes, by the addition of cycloheximide, supports the notion that the reversible effects on rRNA processing is due to hyperthermia directly damaging key enzymes or affecting rRNA secondary structure (Labhart and Reeder, 1987). In addition, Yost and Lindquist (1986) have reported that heat shock reversibly blocks

the splicing of several (and perhaps all) mRNA precursors in *Drosophila* cells causing their stable accumulation in the nucleus. Contrary to Labhart and Reeder (1987) results, cycloheximide inhibition of hsp synthesis during heat shock allows mRNA processing to occur. Yost and Lindquist (1986) concluded that some nuclear hsps (e.g. hsp70 or small hsps) may be responsible for preventing the disruption of mRNA splicing.

Although heat treatment induces drastic changes in transcription, with the immediate inhibition of normal mRNA synthesis and vigorous induction of heat shock mRNAs, these changes alone cannot account for the radical protein synthetic patterns observed. It has become apparent that species-specific translational controls are no less dramatic in achieving rapid shifts in protein synthesis (Lindquist, 1980; Burdon, 1986).

During the heat shock response, the dramatic alteration in the pattern of protein synthesis is accompanied by a parallel change in the distribution of polysomes. Upon exposure to the stress, some mechanism is activated that clears pre-existing mRNAs from polysomes. This is apparent from the simple fact that normal polysomes disappear before heat shock mRNAs accumulate, and disappear even when heat shock mRNA synthesis is blocked. The rapid disassociation of pre-existing polysomes prior to their reformation interrupts protein elongation (translation) of the normal cellular mRNAs (Lindquist, 1980; Storti *et.al*, 1980; Bienz and Gurdon, 1982). Furthermore, new polysomes in HeLa, *Drosophila* fibroblasts and tomato cells have been modified by the dephosphorylation of the ribosomal protein S6. This phosphoprotein is situated at the ribosomal initiating binding site for mRNA and eIF-2 (Glover, 1982; Tas and Martini, 1987). It was thought that the dephosphorylation of S6 was the reason for the change in translational specificity. However, time course experiments using $\alpha^{32}P$ incorporation

proved that translational inhibition occurred faster that S6 dephosphorylation. In addition, results by Tas and Martini (1987) indicated that the dephosphorylation of S6 was not specific for hsp mRNA translation since hsps continue to be translated after reversal of the block.

Eukaryotic translational initiation factors eIF-2α and eIF-4B are phosphorylated and dephosphorylated respectively upon heat shock in HeLa and thymic lymphocyte cells (Duncan and Hershey, 1984; Benedetti and Baglioni, 1986). The phosphorylation of eIF-2α is indirectly accomplished by hsp90 which stimulates the kinases enzyme to phosphorylate eIF-2α (Lindquist and Craig, 1988). The role of these modified factors contributing to the initial inhibition effects of normal protein synthesis is still inconclusive though circumstantial evidence indicates that eIF-2α may in fact be a heat shock protein (hsp39) participating in restoring the cell's ability to initiate protein synthesis (Colbert *et.al*, 1987). Thus it may well be that the phosphorylation of eIF-2α as well as modifications to other initiation factors and ribosomal subunits are all contributors to the translational controls influencing the inhibition and/or recovery of the cells normal protein complement.

The normal complement of pre-existing mRNAs in most species are subjected to translational control during induction of the heat shock response. Pre-existing mRNAs are not degraded but maintained in an inactive masked state, sometimes being sequestered in the cytoplasm (Lindquist, 1980; Storti *et.al*, 1980). Ballinger and Pardue (1983) have shown that pre-existing mRNAs are present in heat shocked *Drosophila* cells associated with ribosomes and RNP particles. Following recovery from heat shock the inactive normal cellular component of mRNA can be translated *in*

vitro or *in vivo* (Storti *et.al*, 1980; Bienz and Gurdon, 1982; Lindquist, 1984).

Yeast cells are an exception in that they do not display translational control of pre-existing cellular mRNAs. Instead most of their mRNAs simply disappear rapidly from the cell while those that are retained continue to be translated (Lindquist, 1980, 1984). This difference in translational control of pre-existing mRNAs between yeast and other species is probably due to differences in their intrinsic mRNA half-lives. Yeast mRNA, having a normal half-life of 20 min to 30 min, will not interfere with the hsp synthesis if the pre-existing mRNAs are allowed to decay at their own rates. In contrast *Drosophila* mRNA has a typical half-life in the order of 6 h to 9 h. Thus to reduce competition for hsp synthesis, transcriptional and translational controls must be implemented to achieve the noticeable and rapid hsp production (Lindquist, 1984).

The specificity of inducible hsp mRNA to be selected and thus preferentially translated seems to reside in the 5' untranslated region of many hsp mRNAs. The unusually long 5' non-coding region (111bp to 253bp) confers selective translation at high temperatures. Deletion studies (5') and hsp/neo chimeric genes containing only the first 32bp of the 5' untranslated leader sequence were transcribed but not translated during heat shock (Ingolia and Craig, 1982b; McGarry and Lindquist, 1985; Spena and Schell, 1987). This long 5' non-coding region is not unique to hsp mRNAs, several murine Hox (homeobox) and proto-oncogenes contain long 5' untranslated leader sequences that my specify translational selection (Dressler and Gruss, 1988). It would seem that specificity for translation of heat shock mRNA may be determined by modified polysomal and initiation factors in

combination with 5' and 3' hsp mRNA regulatory domains (Manrow and Jacobson, 1987).

Finally, restricted organelle HSPs (e.g. grp78 in the ER and small HSPs in the chloroplasts) have been demonstrated to contain a secretory leader peptide. This post-translational precursor sequence is functional for the importation of the HSP protein across intracellular membranes. Upon entry, the leader sequence is lost and the mature and functionally active HSP is restricted to the organelle (Munro and Pelham, 1986; Vierling *et.al*, 1988).

Termination of the Heat Shock Response

The commitment to heat shock synthesis is not a terminal event; when cells are returned to 25^0C normal patterns of transcription and translation are gradually restored. Thus, an equally profound change in specificity of translation occurs during recovery. During recovery there is an unmasking of normal mRNA (with the exception of yeast) and a covalent modification in the protein synthetic apparatus. The rephosphorylation of the ribosomal protein S6 and partial dephosphorylation of eIF-2α may be of considerable importance though these changes do not select against hsp mRNA translation (DiDomenico *et.al*, 1982b; Benedetti and Baglioni, 1986; Burdon, 1986). As one can observe, the complexity of the machinery regulating transcriptional and translational controls is still unclear, though it appears to be specie-specific and dependent upon the severity and type of the stress stimulus.

Developmental and Differentiation-Dependent Inducibility of Heat Shock 70kDa Regulation

In most species, the HSP70-like gene family contains multicopies of both stress inducible hsp and constitutively expressed hsc genes sharing a minimum of 75% homology. The corresponding transcript sizes of 2.7Kb and 2.5Kb respectively, are highly conserved in diverse species (Ingolia *et.al*,

1982; Low and Moran,1986; Mues *et.al*, 1986; Palter *et.al*, 1986). Accumulating evidence has suggested a non-coordinate expression of these proteins in a stage-dependent and tissue-specific manner during embryonic development (Craig *et.al*, 1982; Lindquist, 1984; Heikkila *et.al*, 1986, 1987; Subjeck and Shyy, 1986). Thus, some inducible hsp70s may be expressed constitutively due to developmental regulation under normal physiological conditions (Bensaude *et.al*, 1983; Heikkila *et.al*, 1986) in which even heat cannot induce them (Zimmerman *et.al*, 1983; Morange *et.al*, 1984). This suggests that hsp70 as well as hsc70 proteins may play a functional role in normal embryogenesis.

Variability of HSP70-like expression, concomitant with the activation of the embryonic genome, was observed initially in lower organisms. Monoclonal antibodies to hsc70s revealed that the *Drosophila* hsc70 genes are not coordinately expressed during normal development. These results support the molecular RNA analysis of Craig *et.al* (1983) indicating an equal abundance of the cognate Hsc4 (cytoplasmic hsc70 protein) transcripts in the developing oocyte, embryo, larvae and adult while Hsc1 and Hsc2 were only detected in the adult fly. Existence of translational controls on hsp synthesis (e.g. in *Xenopus* oocytes) must be considered when interpreting stage-specific hsp mRNA synthesis. A distinctive feature of early developmental induction is that hsp68 and hsp70 can be activated by heating only at the blastoderm stage or later, though basal levels in non-heat shocked flies at these corresponding stages have been observed (Lindquist, 1984; Burdon, 1986).

The release of the moulting hormone ß-ecdysterone, has been implicated during insect development in the activation of certain small HSPs. This suggests that the ecdysterone receptor may act as an additional

regulatory element for HSP transcription (Palter *et.al*, 1986; Hoffman *et.al*, 1987).

In mammals, Curi *et.al* (1987) and Morange *et.al*, (1984) have observed that a subset of HSPs are constitutively expressed in the unstressed pre-ovulatory mouse oocytes, spermatids and early embryo. In particular the major heat inducible protein, hsp68, is expressed constitutively only at the two-cell stage although hsc70 expression is evident in the gametes up to the morula stage (Zakeri *et.al*, 1988). It is at the two-cell stage that the activation of the mammalian genome occurs and it has been speculated that the specific transient expression of heat shock genes (i.e. hsp68) may play a role in embryonic gene activation (Bensaude *et.al*, 1983).

At the eight-cell and early morula stage, no spontaneous hsp68 synthesis is observed and stress cannot induce its synthesis until the blastocyst stage or later (Wittig *et.al*, 1983). The induced activation of hsp68 correlates with the acquisition of thermotolerance, thus conferring partial protection from the embryotoxic effects caused by stress agents (Mirkes, 1987). Expression of hsps in mouse embryonal carcinoma cell lines (EC) derived from teratocarcinomas support the evidence of constitutive hsp68 expression obtained from early mouse embryos. EC cell lines propagated as undifferentiated cells are closely related by morphological, biochemical and immunological criteria to the pluripotent cells from pre-implantation mouse embryos. These undifferentiated cells are analogous to pre-implantation embryos in their lack of heat inducible hsp68 synthesis though constitutive levels of hsc70 were observed. As well, a transfected heterologous *Drosophila* hsp70-ß-galactosidase fusion gene could not be heat induced to produce ß-galactosidase activity until EC differentiation (Mezger *et.al*, 1987).

Stress induction of hsp68 was only observed if the EC cell lines, with the exception of F9 EC cells, were caused to differentiate *in vivo* or artificially *in vitro* by retinoic acid (Wittig *et.al*, 1983; Morange *et.al*, 1984; Barnier *et.al*, 1987).

Mezger *et.al* (1987) have proposed a model based on their results obtained with EC cells to account for early mammalian hsp68 expression during embryogenesis. They have speculated that a maternally derived transcription factor(s) triggers the stress independent hsp68 expression in two-cell embryos. Truncation of hsp68 synthesis and its inability to be stress induced at the early cleavage stages (i.e. eight-cell to morula stage) could be related to degradation of the maternal activator or its dilution by the increasing DNA to cytoplasm ratio of the developing embryo. By the onset of the blastocyst formation, the zygotic genome could contribute a functional heat shock gene activator (i.e. HSTF), thus rendering hsp68 heat inducible during differentiation. Other models speculate that the self-regulating HSP70 levels may play a role in modulating developmental stress induction. Furthermore, a DNA binding factor(s) which suppresses transcription during early cleavage, but is diluted to an insufficient level by increased DNA to cytoplasm ratio of the developing embryo cannot be excluded (Heikkila *et.al.*, 1987).

Xenopus embryos also respond to heat shock (i.e. the synthesis of hsps) in a stage-dependent manner reminiscent of the early developmental response of *Drosophila* and mammals. Investigations by Bienz and Gurdon (1982) and Baltus and Hanocq-Quertier (1984) have indicated that hsp70 is stress activated in non-mature oocytes from pre-existing translationally regulated mRNA. Fertilization causes the termination of this heat shock response in the oocytes. The hsp70 transcripts are absent in cleavage stage

embryos and can be detected in older embryos only if induced by heat shock at the mid-blastula stage or later (Bienz, 1984; Nickels and Browder, 1985; Heikkila *et.al*, 1986). The mid-blastula stage of frog development coincides temporally with the activation of generalized zygote transcription.

In situ hybridization studies using a genomic cloned *Xenopus* hsp70 gene has allowed greater sensitivity in the detection of the 2.7Kb hsp70 transcript. Low constitutive levels were apparent in the two-cell embryos while embryos at the mid-blastula or later stages had a constitutive 2.7Kb transcript as well as a heat induced accumulation of a 2.7Kb and 5.0Kb transcript (Browder *et.al*, 1987). Heikkila *et.al* (1987) also reported the presence of a 5.0Kb heat inducible mRNA transcript in preparations from the *Xenopus* neurula. They speculated that it may be an unprocessed transcript derived from one of the four or five *Xenopus* hsp70 genes or a transcript from an as yet unidentified gene which shares homology to the hsp70 family.

Although the main emphasis has been exclusively on the non-coordinate expression of HSP70-like genes during development, though other HSPs have been studied during embryogenesis. It has been shown that a complex spectrum of HSP expression occurs which is either coordinately or non-coordinately regulated with HSP70-like synthesis depending upon the developmental stage. All of these results indicate that the expression of HSPs during embryogenesis may play a major role in developmental differentiation, gene activation and/or cellular protection (Nickells and Browder, 1985; Heikkila *et.al*, 1986, 1987; Subjeck and Shyy, 1986; Krone and Heikkila, 1988).

These modulations in the heat shock response suggest that the synthesis of individual HSPs is regulated independently and is subject to the

unique physiological conditions that exist at each of these transitional stages. Possible explanations for this developmental variability could be any one or a combination of the models outlined above. In addition, a role for the presence of multiple transcriptional control sites of varying affinities for trans-regulating factor(s) cannot be excluded from influencing expression.

Functions of the HSP70-Like Family

Evidence for function is indirect and incomplete. Information comes from a variety of sources; observations made on the survival of normal cells exposed to deleterious conditions, localization of HSPs by immunofluorescence staining, behaviour of site-specific mutants defective in HSP function and the ability to generate transgenic organisms with HSP-fusion chimeric genes.

Despite the fact that the function of the HSPs remains obscure, circumstantial evidence indicates they play a role in the homeostatic control of cellular protection and enhanced survival against non-physiological stress conditions (Burdon, 1986; Bonato *et.al*, 1987; Fujio *et.al*, 1987). Findings from studies employing transcriptional and translational inhibitors demonstrate a correlation between stress protein production and the acquisition of thermotolerance after the stress period. Thermotolerance is the transient acquired resistance to subsequent lethal challenges inherited from an initial non-teratogenic exposure. It is measured as an increase in the critical thermal maximum (CTM): the temperature at which the animal undergoes spasms and becomes unable to escape further exposure to temperatures that would eventually lead to its death (Easton *et.al*, 1987). Thermotolerance allows for a rapid resumption of normal cellular activities during the recovery period (DiDomenico *et.al*, 1982a, 1982b; Subjeck and Shyy, 1986; Mizzen and Welch, 1988). Although there is a strong connection

between HSPs expression and thermotolerance the exact HSPs involved remain only speculative (Lindquist and Craig, 1988). In addition, conflicting evidence by Easton *et.al* (1987) indicate that induced thermotolerance in adult salamanders is independent of HSP induction.

The synthesis of many HSPs can be detected in the normal unstressed cell, thereby implicating their probable participation in cellular processes seemingly distinct from physiological stress (Welch and Suhan, 1986). Stage-dependent, developmental-differentiation of certain HSPs synthesis indicates putative functions during embryogenesis, cell-cycle progression and proliferation (Craig and Jacobsen, 1985, Bonato *et.al*, 1987; van Dongen and van Wijk, 1988). Analogous to the eukaryotes, the prokaryotic hsp70 homologue is important for cell growth (Lindquist and Craig, 1988); perhaps influencing DNA and RNA synthesis. Furthermore, this protein product from the dnaK gene is necessary for the replication of lambda phage DNA during viral infection (Neidhardt *et.al*, 1984; Lindquist and Craig, 1988). It is unknown whether the induced production of hsx70/hsc70 plays a similar role in eukaryotes when they are transfected with the adenovirus E1A gene (Pelham, 1986; Simon *et.al*, 1987).

In order to gain a better understanding of the functions of HSPs, their intracellular locations have been examined. A variety of techniques have been used, including sub-cellular fractionation by centrifugation (Arrigo *et.al*, 1980), direct autoradiographic analysis (Velazquey *et.al*, 1980) and indirect immunofluorescence staining (Welch and Feramisco, 1984; Welch and Suhan, 1986). In general, the small HSPs are found in the nucleus and nucleolus as well as in the plant chloroplasts' photosynthetic membranes (see Table III). HSP70-like proteins are prominent in both the cytoplasm and the nucleus (including the nucleolus) where they bind to the matrix in a salt-

resistant (i.e. hydrophobic) manner (see Table IV). Finally, the HSP83-like proteins are almost exclusively found in the cytoplasm (see Table III). In certain species, HSP70 cytoplasmic localization includes the association with intracellular membranous organelles, the cytoskeleton and ribosomes (Welch and Feramisco, 1984; Baltus and Hanocq-Quertier, 1985; Welch and Suhan, 1986). In addition, grp78 whose basal level can be increased by glucose starvation, is restricted to the lumen of the endoplasmic reticulum (ER), (Munro and Pelham, 1986). Though HSP70's location seems to be dependent upon the type of stimulant, its decline to basal levels in the nuclear region during the reversal of stress treatment is correlated with its decrease in synthesis (Welch and Feramisco, 1984).

Due to its high degree of conservation and its cellular prominence HSP70 has been extensively examined in an effort to elucidate its function. Experiments by Welch and Feramisco (1984) and Mizzen and Welch (1988) using antiserum against hsp72 and hsc73 in indirect immunofluorescent studies on rat fibroblasts indicated an apparent integral role for HSP70 in the acquisition of thermotolerance. Observations that reinforce the possible connection of HSP70 levels with the relative intrinsic degrees of thermotolerance came from certain cell lines that are heat resistant. These heat resistant cells (e.g. HeLa cells) contain constitutively expressed HSP70 proteins at 37^0C giving them a higher intrinsic thermotolerance at subsequent 43^0C heat incubations. Furthermore, developmental regulation of inducible HSP70 synthesis has been correlated with acquisition of thermotolerance during embryogenesis (Mirkes, 1987).

Recent work by Welch and Mizzen (1988) revealed the redistribution of pre-existing hsp72 and hsc73 from the cytoplasm and nucleus into the nucleolus after a second and more severe heat shock treatment. This

migration and their subsequent exit was markedly faster in the mammalian heat tolerant cells as compared with the non-tolerant cells. Furthermore, the translocation of the pre-existing HSP70 proteins were shown to be dependent upon heat shock treatment and independent of active HSP70 synthesis. Immunoelectron microscopy and silver staining localized hsp72 to the granular region residing within the nucleolar-organizing centre. This is the site within the nucleolus in which pre-ribosomes and some other ribonucleoprotein complexes are assembled (Welch and Suhan, 1986).

It appears at the moment that non-physiological conditions cause the redistribution of pre-existing and the induction of new HSP70 proteins. These proteins are involved in conferring cellular protection from the teratogenic effects of hyperthermia. HSP70s might serve in repair and/or recovery of nucleolar function which is known to be perturbed after heat shock treatment (Welch and Feramisco, 1984; Welch and Suhan, 1986). The presence of some HSP70 proteins associated with ribonucleoprotein complexes containing poly(A)$^+$ heterogeneous RNA may interfere with the processing of mRNA precursors. Large unprocessed RNA molecules derived from the normal cellular transcripts have been observed by Yost and Lindquist (1986) and Labhart and Reeder (1987). Cytoskeleton association with HSP70 proteins may ensure structural integrity while ribosomal and RNA cytoplasmic binding might facilitate stabilization and rapid translational recovery after stress termination (DiDomenico *et.al*, 1982a, 1982b, Baltus and Hanocq-Quertier, 1985). Welch and Mizzen (1988) suggest that thermotolerance acquisition and the higher degree of intrinsic heat tolerance may be partially attributed to the constitutive presence of the cognate 70 proteins and basal hsp70 levels.

An important clue to the mechanism of action of HSP70 proteins came from evidence by Welch and Feramisco (1985) that HSP70s contain ATP-binding sites. Additional evidence by Chappell *et.al.* (1986) implementing genetic and immunological techniques identified the constitutive cognate 70kDa stress protein as an ATP dependent "uncoating ATPase." This enzyme plays a role in biosynthesis by removal of the protein clathrin from vesicles which are intermediates in the pathway of receptor-mediate endocytosis. Hsc70 binds to the hydrophobic regions of clathrin in the presence of ATP which acts as a catalyst. The subsequent ATP hydrolysis dissociates the clathrin interactions allowing for the fusion of the enclosed membrane-proteins to the appropriate plasmalemma (Schmid and Rothman, 1985; Chappell *et.al.*, 1986; Rothman and Schmid, 1986).

It is unlikely that uncoating is the only function of hsc70 since hsc70 binds to the nucleus and nucleolus after heat shock. Finally, grp78 which is not induced significantly by high temperature has been shown to be an ATP-binding protein indistinguishable from the immunoglobulin heavy chain binding protein (BIP), (Munro and Pelham,1986). Thus the role of HSP70 proteins is an apparent ATP dependent reaction that may be reversed upon hydrolysis of ATP.

A hypothesis was put forth by Minton *et.al.*, (1982), which suggested that HSPs might contribute to enhanced thermotolerance in cells by non-specifically stabilizing stress-susceptible proteins. Pelham (1986) proposed that the HSP70 family with its general ability to disrupt protein-protein interactions (as observed by hsc70 interaction with clathrin) was the most likely candidate to perform this specific task. The proposed function of HSP70 is to solubilize aggregated, stress damaged proteins in an ATP dependent manner, particularly in the nucleus and nucleoli.

Pelham's tentative model (Figure IV) suggests that hydrophobic sites of proteins are exposed, due to stress damage, and interact to form insoluble aggregates. HSP70 proteins, catalyzed by ATP, bind tightly to these hydrophobic regions thus limiting their interactions and promoting disaggregation. Hydrolysis of the ATP disassociates HSP70 and induces a conformational change in damaged protein that was bound. This distorted change in protein conformation may now be able to refold or reassemble into its native form (Pelham 1986).

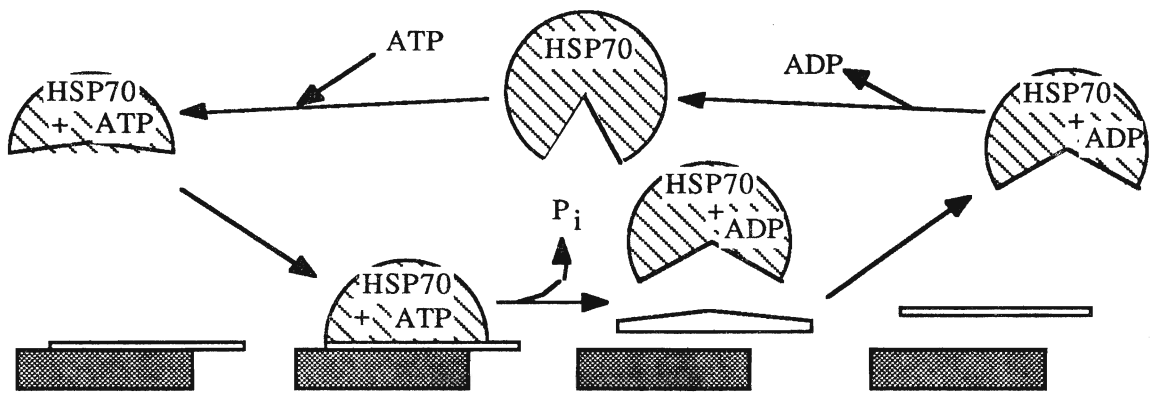
Support for the above model has been found in the mammalian ER where basal levels of grp78 are localized. Recently, grp78 has been found to play an intregal role in the stability of nascent and unfolded proteins in the lumen of the rough ER. Glucose deprivation, an inducer of grp78 levels, inhibits N-linked glycosylation of nascent proteins in the ER. It is thought that the undergylcosylation and/or the exposure of hydrophobic domains in malfolded proteins can be stabilized by the reversible binding of the ATP dependent grp78 (Sharma *et.al*, 1985; Gething, *et.al*, 1986; Munro and Pelham, 1986; Kozutsumi *et.al*, 1988). In addition, the yeast hsc72 protein, encoded by the Hsc3 gene, has been implicated as being analogous to the mammalian glucose-regulated protein, grp78, in its function (Lindquist and Craig, 1988).

It is interesting to note HSP70s' affinity for stress altered aberrant proteins since it has been proposed that the proteolytic system has a role in HSP induction (see Figure III). Pelham (1986) concluded that the feedback inhibition of HSP70 could be explained by the renaturation or protection of denatured proteins.

The existence of HSP70 proteins in normal, unstressed cells suggest that they are important for normal cellular metabolism. HSP70s may

Figure IV. A postulated model of HSP70's mechanism of action. Aberrant proteins induced by stress agents form insoluble aggregates that bind ATP-modified HSP70 via hydrophobic interactions. Hydrolysis of ATP causes the release of HSP70 and conformational change of the protein aggregates. The released substrate may refold or reassemble into its native, pre-heat shock state.

Figure IV. Stabilization of Denatured Protein Aggregates



(Pelham, 1986)

recognize nascent proteins in the cytoplasm (which by definition are denatured), and sort out any aggregation problems that occur during their folding and assembly into oligomeric structures. The preferential recognition and binding of HSP70 to an incomplete oligomeric protein molecule may provide a temporal and stabilizing period that will exist until additional protein subunits are synthesized and bound to complete the protein polymer. Functions of this kind would be consistent with the high concentration of hsc70 observed in rapidly proliferating cells; a period characterized by increased levels of protein synthesis (Pelham, 1986; Ferris *et.al*, 1988; Haire *et.al*, 1988; van Dongen and van Wijk, 1988). Implication of grp78 in the normal assembly of secreted and membrane-bound proteins is supported by the fact that grp78 is identical to the immunoglobulin heavy chain binding protein (BIP). The protein BIP (located in the ER of lymphocytes) binds to the hydrophobic regions of nascent Ig heavy chains that are not yet associated with their light chain counterparts. Reversible binding of BIP or grp78 will prevent or reverse the formation of heavy chain aggregates, hence aiding the process of immunoglobulin assembly (Munro and Pelham, 1986; Kozutsumi *et.al*, 1988).

Site-specific mutations in *Saccharomyces cerevisia* 70kDa cognate and inducible genes has led to the conclusion that HSP70 gene products are essential for growth and viability at all temperatures, indicating a critical role in normal cellular physiology for the encoded proteins (Craig and Jacobsen, 1985; Bonato *et.al*, 1987; Craig *et.al*, 1987). Although the nature of their modulated expression and function during cell growth at different temperatures is just beginning to be understood by mutant strains (Lindquist and Craig, 1988), it has become evident that some yeast HSP70 genes and their corresponding proteins are unique and cannot be

complemented by related family members (Bonato *et.al*, 1987; Craig *et.al*, 1987).

Adding further complexity to the variable functions of HSP70 proteins (and other HSPs) is their induction in tumours and their relationship to oncogene products. The tumour-associated nuclear phosphoprotein, p53, is an oligomeric complex that can induce immortality in primary cell cultures or generate foci when cotransfected with the ras oncogene. In the transformed state, high levels of p53 and hsc70 are produced. These two proteins combine in a 5:1 ratio (p53:hsc70) to form a 660kDa molecule (low affinity to hsp70 by p53 has been observed). This molecule interacts with another 110kDa component at the hsc70 domain. The reversible interaction with the hsc70 protein, stabilizes the rapid half-life of p53 in much the same way as the large T antigen-p53 complex does in SV40 transformed 3T3 cells. At this time, the significance of the p53-hsc70 complex in contributing to the transformed neoplastic state is still unclear (Clarke *et.al*, 1988; Lindquist and Craig, 1988).

It is interesting to note that *E. coli*'s hsp70 (B66.0, product of dnaK gene) protein has affinity to bind to the ligand, p53. This parallel, among others, has lead to the speculation that hsc70 regulates p53 levels in an analogous fashion to the proposed dnaK gene product regulation of σ^{32} in *E. coli* (Clarke *et.al*, 1988).

As with p53, c-myc and c-myb have been categorized as nuclear oncogenes which play a role in the control of cell proliferation and differentiation. The c-myb oncogene is a nuclear phosphoprotein that contains DNA binding properties (Luscher and Eisenman, 1988). Work by Luscher and Eisenman (1988), revealed a strong correlation in c-myc and c-myb half-life stability (five-fold and two-fold, respectively) after heat

treatment. Heat shock also had the effect of increasing the rate of c-myc protein synthesis two-fold, whereas c-myb translation was decreased nearly four-fold. It is plausible that the stability of the oncogene proteins and their translational regulation is related to their association with HSP70 proteins.

Does heat shock affect transcriptional levels of c-myc and c-myb? If so, are there any promoter homology (i.e. HSEs) between these oncogenes and the HSP70 genes? Precedence for serum regulation (via SRE) has been found in the nuclear oncogene, c-fos, as well as in HSP70 promoter genes (Treisman, 1987; Wu *et.al*, 1987). It would be of interest to analyze c-myc and c-myb mRNA expression after heat shock in addition to their 5' promoter sequences and protein binding regulatory domains.

Recent work with synchronized cultured human lymphocytes (G_0) have demonstrated that a mitogen (phytohemagglutinin) and a serum growth factor (interleukin-2) can induce the transcription of hsp70 (two-fold) and increase the level of hsc70 (four-fold) by the G_1/S phase (Ferris *et.al*, 1988; Haire *et.al.*, 1988). It was not unexpected that serum would induce hsp70 expression as Wu *et.al* (1986, 1987) located a separate serum domain upstream from the HSE regulating domain. Though these results are relevant to developmental regulation of mitogenesis and cell proliferation of human lymphocytes, contradictory results have been obtained by Kaczmarek *et.al.* (1987).

Functions of the Small HSPs

In regard to the small HSPs, their intracellular distribution and function appear to vary among different organisms or cell types. Yeast cells in particular, contain only one small HSP and mutations of this gene have failed to identify any aberrant phenotypes. Generally, small HSPs play a role in cellular protection with emphasis on nuclear maintenance. In

Dictyostelium the small HSPs are chromatin associated while in the *Drosophila* their nuclear accumulation was found in combination with the nuclear scaffolding (Burdon, 1986; Subjeck and Shyy, 1986). This protective mechanism of the small HSPs also occurs in plants (Vierling *et.al*, 1988). Schuster *et.al.* (1988) emphatically specified that the small HSPs function to protect against photoinhibition of photosystem II's reaction centre during heat stress.

Early speculation of small HSPs involvement in thermotolerance acquisition and chromosomal regulation is in dispute due to recent evidence of HSP70 expression during embryogenesis (Burdon, 1986; Subjeck and Shyy, 1986). The tendency for small HSPs to aggregate into large proteins, in much the same manner as the partially homologous α-crystallin proteins, may indicate some biological activity that has yet to be unravelled. In addition, the small hsps share the property of being induced at specific stages in development at normal temperatures and thus may have a role in embryogenesis (Lindquist and Craig, 1988).

Functions of the HSP83-Like Family

The exception to the cytoplasmic distribution of HSP83-like proteins is the mammalian 110kDa heat shock protein. This protein unlike the inducible mammalian hsp68 is present in the nucleolus even in the absence of stress. The function of this 110kDa protein is unknown though it binds to the fibrillar structure (site of rDNA) as well as to RNA (assayed by its release after RNase treatment) (Subjeck and Shyy, 1986).

Probably the most distinctive feature of the cytoplasmic HSP83-like proteins is their regulatory roles via steroid receptor association. These steroid receptors are trans-acting regulatory factors that bind to and activate the steroid dependent gene. The genes themselves that encode for the

receptor proteins are autoregulated by hormone complexes (Maniatis *et.al*, 1987; Green and Chambon, 1988). Steroid receptors in the cytosol exist in an inactive, non-DNA-binding form that can be transformed via hormonal (e.g. progesterone, estrogen, androgen, and glucocorticoid) and temperature-dependent manner to its active DNA binding state. Sanchez *et.al.* (1987) and Picar *et.al.* (1988) have demonstrated that the inactive steroid receptor is associated with a HSP90 protein, forming a large 300kDa complex, that inhibits activation by steroid binding. By some unknown mechanism HSP90 dissociates in transformed cells thus allowing for hormone interaction and the production of a functional DNA binding regulating factor. Most steroid trans-acting factor contains zinc (Zn^{2+}) binding domains and its ability to bind to DNA seems to be dependent on Zn^{2+} interacting with the zinc finger motifs (Sabbah *et.al*, 1987; Dressler and Gruss, 1988).

HSP83-like proteins have also been implicated in conjunction with a 50kDa protein to be transiently associated with the Rous sarcoma virus transforming protein, $pp60^{v\text{-}scr}$, maintaining $pp60^{v\text{-}scr}$ inactivation during its passage to the plasma membrane. This tyrosine kinase associates with hsp90 and a 50kDa phosphoprotein immediately after it is synthesized. At or about the time it is released from association with hsp90, it is phosphorylated on tyrosine, inserted into the membrane, and activated as a kinase. Further evidence has found five other transforming proteins with tryrosine kinase activity that form stable complexes with hsp90 and a 50kDa protein (Lindquist and Craig, 1988).

Contradictory evidence of an HSP having poly(A)$^+$ affinity has been attributed to the HSP83-like class. This protein may demonstrate mRNAs masking property in the sequestering and inactivation of normal cellular

mRNAs which occurs during stress treatment (Subjeck and Shyy, 1986; Bardwell and Craig, 1987; Rebbe *et.al*, 1987).

Methods and Materials

All procedures were conducted under sterile conditions. Solution compositions are described in the Appendix.

Newt Maintenance

Adult red-spotted newts (*Notophthalmus viridescens*) purchased from Mr. C. Sullivan, Nashville Tennessee; were housed in aquarium tanks (160L) containing distilled water. Fresh water was exchanged daily and maintained at approximately 19^{0}C. The animals were fed live Tubifex worms twice weekly and washed briefly in 0.1% potassium permangenate and rinsed in dH_2O the following day.

Tissue Samples

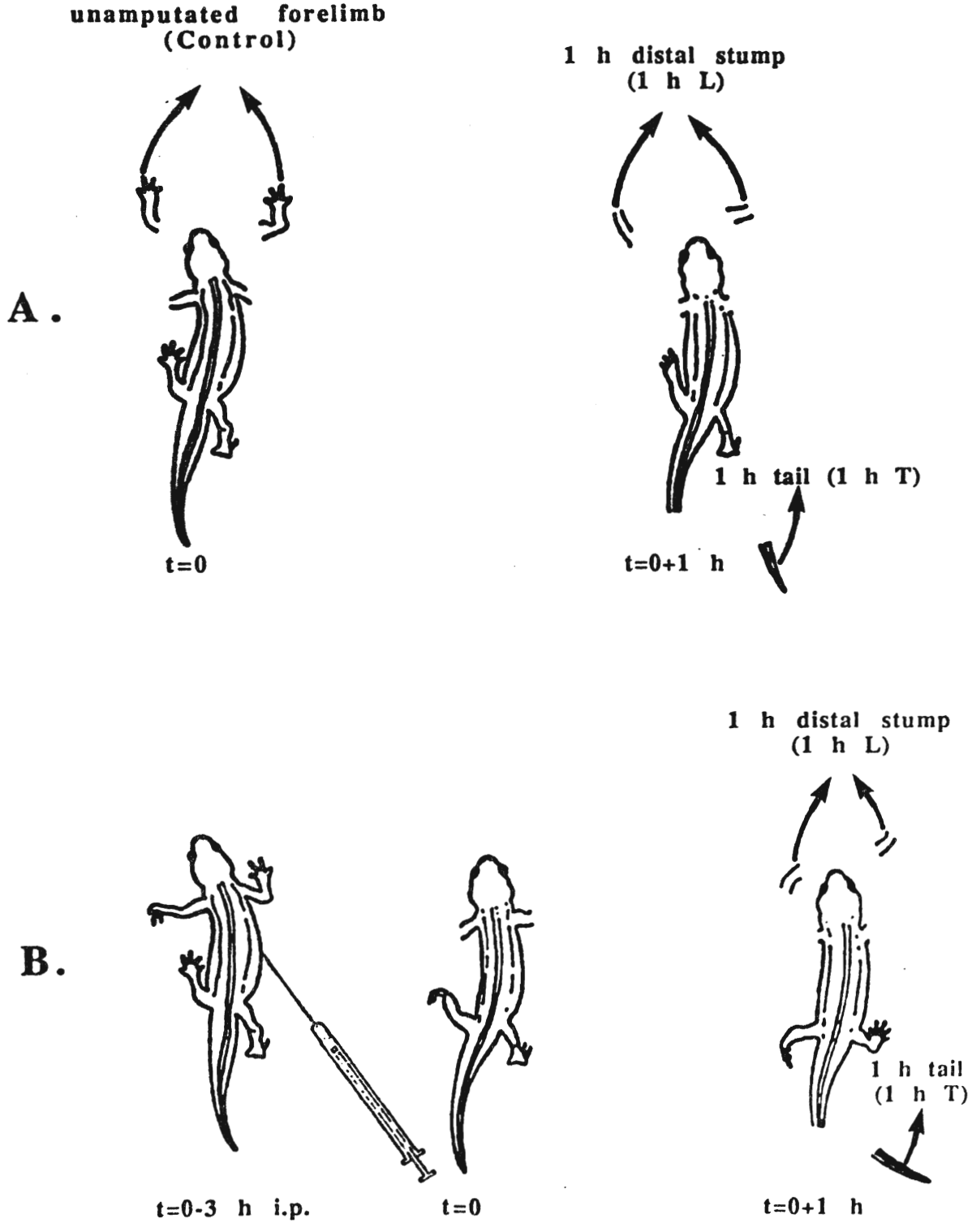
a) Protein Analysis

Tissues corresponding to the morphological phenotypes of early bud (E.B.), latebud (L.B.) and early digit (E.D.) were excised from newt regenerates and frozen in liquid N_2. Forelimbs bilaterally amputated through the distal humerus as well as a 10mm section of the distal tail were obtained and frozen from both the 19^{0}C control and the 1 h 34^{0}C heat shock/0.5 h 19^{0}C recovery animals. *Notophthalmus viridescens* was found to have a CTM of approximately 36^{0}C similar to that found by Easton *et.al* (1986) and Rutledge *et.al* (1987) for *Pleurodeles sp.* Distal 3mm limb stump tissues, of post-amputated forelimbs (t=0), and unamputated tail tissues were collected from individual newts and frozen separately at time periods (t=0+1 h, t=0+2 h, t=0+6 h, t=0+1 d and t=0+2 d) as shown in Figure V.A. Tissues from three newts were pooled for each experimental sample.

Radiolabelling of proteins was accomplished by intraperitoneal injection (i.p.) of 100µCI/20µl Trans ^{35}S-label^{TM} L-methionine and L-cysteine (ICN, sp.act.=1005Ci/mmole). Singer and Caston (1972) reported

Figure V. Drawings depicting collection and immediate freezing of appendages from anesthetized newts. (A) represents primary bilateral forelimb amputation (t=0) and collection of distal limb stump and tail tissues 1 h (t=0+1 h) post-forelimb amputation (p.f.a.). (B) represents collection of radiolabelled distal stump and tail tissues (1 h p.f.a.). Newts were injected intraperitoneally (i.p.) with 100µCi ^{35}S-methionine (t=0-3 h i.p.) and primary bilateral forelimb amputation was done 3 h later (t=0). Distal limb stump and tail were isolated 1 h later (t=0+1 h) for a total of 4 h ^{35}S-methionine incorporation.

Figure VA/B. Schematic Representation of Tissue Collection



that incorporation of intraperitoneally injected ^{35}S-methionine in newts was linear from 1 h to 4 h after injection. In this study, incorporation of the radioactive amino acids was 4 h for the control, heat shock, and post-amputation newts. For example, the unamputated control newts were injected with Trans ^{35}S-label 4 h (t=0-4 h i.p.) prior to primary bilateral forelimb excision (t=0) while the heat shocked newts were injected 1 h before the 1 h/34^{0}C water-bath immersion and amputated at the end of a 2 h/19^{0}C recovery for a total of 4 h Trans ^{35}S incorporation. The 1 h post-amputation samples (Figure VB) were injected 3 h (t=0-3 h i.p.) prior to the primary bilateral humerus amputation (t=0) and the 3mm distal stump and distal tail sections were excised and collected 1 h later (t=0+1 h).

All tissue samples were immediately frozen in liquid N_2 until their proteins were isolated and concentration determined as outlined in "Protein Polyacrylamide Electrophoresis". Alkali resistant, trichloroacetic acid precipitation assays were preformed to determine ^{35}S L-methionine and ^{35}S L-cysteine incorporation (see TCA Protein Precipitation).

b) Molecular Analysis

Prior to amputation, the newts were anesthetized by immersion into 0.03% ethyl-methylbenzocaine (dissolved in dd$H_2$0) for approximately 15 min and rinsed in 0.1% gentamycin. Forelimbs were collected for DNA analysis and immediately frozen in liquid N_2 after bilateral amputation through distal third of the humerus. Stump tissues were clipped of any protruding humerus to prevent soft-tissue retraction prior to recovery and regeneration.

Initially, tissue isolation for mRNA analysis was conducted as outlined under protein analysis. Mechanical difficulties with the -70^{0}C freezer caused the degradation of RNA isolated from over 100 pooled tissue samples. Thus

time and monetary constraints allowed for RNA extraction from whole animals only. Anesthetized newts were exposed to either: (1) a 34^{0}C water-bath heat shock for 1 h with a 15 min recovery at 19^{0}C or (2) forelimb and hindlimb bilateral amputations through the distal third of the humerus and recovery for 2 h or (3) immersion in for 1 h at 19^{0}C in dH_20 to act as a control. Upon completion of the experimental manipulation, the newts were decapitated and immediately frozen in liquid N_2 prior to total body RNA isolation.

Protein Polyacrylamide Electrophoresis

In preparation for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), frozen tissue samples were homogenized on ice in micro-homogenizers containing 250µl of 1xTE buffer, pH6.8. The transferred homogenate was spun for 2 min in a Fisher microfuge (4^{0}C) and the supernatant decanted to fresh Eppendorfs. Protein concentrations using BSA as a reference were determined by the Lowry method (Scopes, 1982) using the Beckman DU-50 spectrophotometre (600nm) equipped with a protein assay soft-pac^{TM} module.

Discontinuous SDS-polyacrylamide slab gels were built using BioRad's Protein II Multi-cell (20cm x 18cm x 1.5mm) or Mini-Protein II (8cm x 5cm x 0.75mm) apparati. A 4% acrylamide stacking gel (0.1% SDS, 0.125M Tris, pH6.8) was overlaid onto a polymerized 10% or 12% separating gel (0.1% SDS, 0.375M Tris, pH8.8). Both the stacking and separating SDS gel solutions were prepared from a stock solution of Acrylamide/Bis (30% T, 2.67% C) that had been degassed 15 min prior to the addition of 0.05% ammonium persulfate (AMPS) and 0.005% TEMED (as per BioRad's manual).

Protein samples were diluted 1:4 with SDS sample buffer and allowed to sit for 1 h at room temperature preceeding further denaturation at 95^{0}C

for 4 min. Samples were loaded into the wells and the voltage or current adjusted for protein separation depending upon the apparatus used. Pre-stained SDS-PAGE standards (BioRad) were electrophoresed with the protein samples to determine molecular sizes. Electrophoresis with the Protein II Multi-Cell was at 25mA/gel, constant current, until the bromophenol blue dye marker entered the separating gel and then the current was raised to 35mA/gel. A cooling core of flowing cold water was applied during the protein separation. The Mini-Protein II was run under constant voltage (200V) and electrophoresis was carried out until the dye marker left the bottom of the separating gel. Upon completion the apparatus was disassembled and the gel stained with 0.1% Coomassie Blue B in fixative (40% MeOH/10% HOAc) for 30 min followed by destaining in the fixative alone. Relative percentages of integrated peak areas were determined by the use of a digital planimetre (Placom) tracing the densitometre scans (Helena Auto Scanner containing a 570nm filter) generated from the stained gels.

Western Blot Analysis

Western blot analysis was performed as outlined in BioRad's Immun-Blot™ Assay Kit. This is an enzyme immunoassay kit (alkaline phosphatase) that identifies the antigen-antibody immune complexes by colour development.

Protein samples of equal amounts and volumes were subject to SDS-PAGE using the Mini-Protein II apparatus (see Protein Polyacrylamide Electrophoresis). The polyacrylamide gels were not stained/destained until after electrophoretic transfer of the proteins to a nitrocellulose (NC) filter. Prior to transfer, gels of known orientation were equilibrated twice in 4^0C transfer buffer (25mM Tris, pH8.3/192mM Glycine/20% MeOH [v/v]) for

30 min. Equilibration allows for the partial renaturation of the proteins and facilitates SDS removal from the gel. Transfer was accomplished in transfer buffer overnight at 4^0C using BioRad's Trans-Blot and Model 250/2.5 Power Supply apparati (30V, 0.1A). The next day, voltage and current were increased (70V, 0.25A) for 3 h to enhance protein transfer. Efficiency of protein transfer was determined by physical staining with Amido Black on replicate NC blots.

The NC blots were then rinsed twice for 10 min in TBS (20mM Tris/500mM buffered saline, pH7) followed by blocking of non-bound protein sites by the addition of 3% gelatin/TBS (3 h with agitation). The NC was washed 2x with TTBS (0.5% Tween-20/TBS) (30 min each) to eliminate overall back-ground binding and non-specific hydrophobic interactions. The membrane was transferred from TTBS to a solution containing a 1:200 dilution of the primary antibody (see Probes and Antibodies) and incubated overnight in antibody buffer with gentle rocking. The next morning the unbound primary antibody was removed by three 30 min washes in TTBS followed by a single 30 min wash in TBS. The goat anti-mouse alkaline phosphatase conjugate (GAM-AP, BioRad Immunoassay Kit) IgG secondary antibody (1:3000 dilution) was applied in antibody buffer for 1 h with agitation. The NC was then washed twice in TTBS for 30 min followed by 30 min in TBS. Colour development was achieved by immersion of the NC membrane into BCIP/NBT Alkaline Phosphatase conjugate solution (BioRad) and stopped by transferal of the membrane to ddH_20 for 10 min.

N.B. Testing of non-specific secondary antibody binding was controlled for by the elimination of the primary antibody incubation from a electrophoretically blotted NC replicate.

TCA Protein Precipitation

Two protocols were used to determine the radioactive incorporation of Trans ^{35}S L-methionine and L-cysteine into isolated proteins. The combined average cpm/µl of the two methods was used to load equal sample counts (cpm) or amounts (µg) per lane on electrophoretic gels. Standard deviation was calculated for the combined average cpm/µg from three separate experiments.

a) Method A

Five microlitres of the radiolabelled protein mixture was placed into a 10ml polystryene tube (Falcon) to which 1ml of 1N NaOH/1.5% H_2O_2 solution was added. The reaction was allowed to proceed at 37^0C for 10 min prior to the addition of ice cold 25% TCA/2% casamino acids (4ml) and the solution incubated on ice for 30 min.

The precipitate was collected by suction on Whatman GF/C fibre filters using a Millipore apparatus. The Falcon tubes were washed twice with 10ml 8% ice-cold TCA and loaded onto the corresponding filters. The filter were washed again with a few millilitres of acetone under vacuum and baked dry in scintilation vials at 60^0C prior to the addition of 10ml aqueous counting scintilant (Amersham). The incorporation of Trans ^{35}S-label was measured by using a Beckman LS 1800 scintillation counter.

b) Method B

Another 5µl aliquot of the radiolabelled protein mixture was placed into an Eppendorf containing 50µl BSA (1mg/ml) and 50µl methionine (1mg/ml). To this was added 1ml ice-cold 20% TCA and the Eppendorf was mixed by inversion before being precipitated on ice (30 min).

The precipitate was collected as in Method A above. The Eppendorf was washed twice with 1ml cold 8% TCA and applied to the corresponding

filter. The filter was sequentially washed with ice-cold 8% TCA (10ml) and 95% EtOH (10ml) prior to drying and its incorporation determination as described in Method A.

Fluorography (Fluorogram)

This procedure facilitates autoradiography of radiolabelled materials separated by gel electrophoresis. The protocol was followed as outlined by NEN research products guide.

Radiolabelled proteins samples of equal counts (cpm) or amounts (µg) were loaded and electrophoresed under conditions previously described (see Protein Polyacrylamide Electrophoresis). After staining/destaining in fixative the gel was impregnated by constant agitation for 1 h with EN^{3}HANCE (NEN) in a polyethylene tray. The fluor in the gel was precipitated by immersion of the gel in cold water for 1 h with agitation. The used enhancer solution was discarded into an appropriate radioactive waste container.

The gel was placed onto a piece of 3M Whatman filter paper and transferred to a slab gel dryer (BioRad, Model 543). Saran wrap was stretched over the gel and all bubbles were expelled by rolling the top of the gel with a pasteur pipette. The gel was dried at 60^{0}C for approximately 1 h and the saran wrap removed prior to exposure. The dried gel was exposed to Kodak X-Omat AR film in a Wolf cassette contaning Cronex intensifying screens. The cassette was placed at -80^{0}C for a minimum of 48 h before developing.

The film was developed in Kodak GBX Developer and Replenisher at 20^{0}C for 5 min. The film was then rinsed in a water stop bath for 30 sec and fixed at 20^{0}C for 4 min in Kodak GBX Fixer and Replenisher. The exposed film was again washed in a water-bath for 5 min and then allowed to dry. The fluorograph of replicate experiments showing the highest resolution of bands

was scanned by a densitometry (Helena Auto Scanner, visible light). Relative percentages of integrated peak areas were determined by the average of three digital planimetre (Placom) tracings generated from densitometre scans of the exposed film.

Two-Dimensional Fluorography

This procedure was a modification of the methods of O'Farrell *et.al.* (1977) and Garrels (1979). The first dimension involves isoelectrofocusing (IEF) during which proteins are separated according to their isoelectric points. The second dimension is SDS-polyacrylamide gel electrophoresis. During this step proteins or subunits are separated by molecular size. Protein concentration and radioactive Trans ^{35}S incorporation were determined as previously described.

To an Eppendorf containing 100µl of radiolabelled protein sample was added 10µl of SDS/ß-ME. The mixture was vortexed briefly and placed on ice for 1 min prior to a quick freeze in liquid N_2. The protein solution was lyophilized overnight in a Labconco Freeze Dryer and the dried sample was resuspended in 100ul of 2DSß and dissolved at 37^0C for a few minutes. Polonged warming is inadvisable due to the potential decompositon of the urea causing carbamylation of the proteins. The mixture was frozen in liquid N_2 and stored at -80^0C until used. It is critical to maintain the volume ratio of 2SDSß to SDS/ß-ME at 10:1. The final concentration of SDS (0.3%) is important; lower or higher concentrations of SDS will alter the pH profile.

The isoelectric focussing gels (IEF gels) were composed of ampholytes 3.5-9.5/6-8 (LKB) to generate a pH gradient. The pH range was fortified by the addition of 6-8 ampholytes to reduce the extensive buffering in the neutral pH range induced by urea. The IEF gel solution was degassed (15 min) and mixed by inversion after the addition of 8µl of 10% APS. Seven

centimetre gels were poured by use of a tube gel loading needle into acid washed and ddH_20 rinsed 10cm glass tubes (1.5mm I.D.). The IEF gels were polymerized for 1 h at room temperature and only those gels not containing air bubbles were loaded into the BioRad Tube Cell apparatus (Model 175).

Freshly made degassed electrolyte buffers (catholyte and anolyte) were poured into their corresponding compartments to a depth that covered the tube openings. Pre-warmed (37^0C) overlay solution (4µl) was applied to the top of the gels with a Hamilton syringe. The gels were equilibrated by pre-focussing at constant current until the voltage increased from 300V to 1000V. Pre-focussing establishes the pH gradient, removes the APS, and increases the resistance of the gels so that they can be run at 1000V. Radiolabelled protein samples (10µl) of equivalent counts (cpm) were warmed (37^0C) and layered below the residual overlay solution, i.e. directly on the top of the gels.

The tubes were focussed at 1000V (constant current) for 6 h and quick fixed with dry-ice after completion of electrophoresis. Duplicate gels were extruded from the tubes upon completion into IEF gel reducing buffer (10 min) or Coomassie stained/destained fixative (see Protein Polyacrylamide Electrophoresis). Non-stained equilibrated IEF gels were layer directly onto the second dimension, discontinuous slab gel (4% stacking, 10% separating) for molecular weight separation (see Protein Polyacrylamide Electrophoresis). Fluorography of the second dimension was conducted as previously outlined (see Fluorography) with the exception that the slab gels were not stained with Coomassie blue. Fluorograms were subjectively compared by overlaying two exposures on a light box and aligning prominent and common migrating reference spots. Silver staining of the gels

was conducted after X-ray film exposure by rehydrating the gels in water and following BioRad's silver kit protocol.

Isolation of High Molecular Weight DNA

Newt forelimbs and tissues from other organisms were isolated and homogenized individually in micro-homogenizers containing 700µl of Proteinase K Buffer and then transferred to Eppendorf tubes. To each homogenate, 35µl of a 10mg/ml stock of Proteinase K was added and incubated at 55^0C overnight with gentle rocking. Upon completion, 20µl of preheated RNase A (10mg/ml) was added and allowed to incubate at 37^0C for 2 h.

To the emulsion, 0.5ml P:C:IA was added and allowed to gently rotate for 20 min prior to the separation of the aqueous and non-aqueous mixture by microcentrifugation (10 min). The aqueous and protein interphase was collected and transferred to new Eppendorfs to which 10µl of RNase A was added. The solution was incubated, extracted with P:C:IA, and separated as previously described. The organic phase only was transferred to a new Eppendorf and twice extracted with C:IA (24:1). Finally, the aqueous phase was again transferred to a new Eppendorf and the DNA precipitated at room temperature with isopropanol.

The DNA pellet was microfuged for 30 min at 0^0C, washed with 70% EtOH, dried in a Savant speed vac concentrator with heat and dissolved in 1xTE, pH7.2. Concentration and purity of the DNA was determined by spectrophotometrically (see Large-Scale Isolation of Plasmid DNA) while the integrity of the high molecular weight DNA was determined on a 1.5% agarose mini-gel containing ethidium bromide. The DNA samples were stored at 4^0C.

Newt RNA Isolation

Newt RNA extraction was performed by modifying the guanidinium isothiocyanate (Gu.Iso.) isolation procedure described by Maniatis *et.al.* (1982).

All glassware for isolation of RNA and poly(A)$^+$ mRNA was soaked in a 0.1% solution of diethylpyrocarbonate (DEPC) for a few hours and subsequently baked at 200^{0}C overnight. All solution (except Tris buffers) were made fresh in 0.1% DEPC.dd$H_2$0 and autoclaved.

Experimentally manipulated newts were ground by a pestle in a pre-cooled, liquid N_2 containing mortar. The tissue powder was transferred to Falcon tubes (2057) containing 7ml Gu.Iso. buffer and mixed thoroughly. High molecular weight DNA in the solution was sheared by several passes threw a 22 gauge needle attached to a 20ml syringe. Cesium Chloride (1g per 2.5ml homogenate) was added and the dissolved RNA-$CsCl_2$ solution was overlaid on top of a 3ml 5.7M $CsCl_2$ cushion in a SW41 polyallomar centrifuge tube.

Balanced tubes were centrifuged at 30000rpm, 20^{0}C for 48 h in an IEC SB-283 rotor. The supernatant was carefully aspirated off and the RNA was evident as a glistening pellet at the bottom of the tube. The RNA was allowed to air dry and resuspended in 400µl TES. To eliminate melanin contamination, the RNA solution was microcentrifuged in Eppendorfs for 2 min at room temperature and the supernatants transferred to fresh Eppendorfs. To each tube, 0.1 volume of 3M NaOAc, pH5.2 and 2 volumes of 95% ethanol (-20^{0}C) were added and left to sit overnight at -20^{0}C.

The RNA precipitate was isolated by microfuge at 4^{0}C for 15 min. The supernatant was aspirated and the RNA pellet washed in 70% ethanol and

dried in a Savant speed vac concentrator without heat prior to being resuspended in 200µl DEPC.ddH_2O. Concentration and purity was determined spectrophotometrically (1.0 $O.D._{260nm}$ = 40µg/ml RNA) while RNA integrity (i.e. visualization of ribosomal RNA 28S, 18S and 5.8S) was determined on a 1.2% TBE agarose mini-gel after incubation for 15 min in a 60^{0}C H_2O bath. The RNA samples were stored at -70^{0}C.

Selection of Poly(A)$^+$ mRNA

To a disposable poly-prep column (BioRAD) oligo(dT)-cellulose in sterile DEPC.ddH_2O was added at a concentration of 0.125g oligo(dT)/mg RNA. The column was pre-washed with twenty column volumes of 0.1M NaOH/0.01M EDTA solution prior to the addition of binding buffer (Bb$^+$). Twenty column volumes of binding buffer (0.4M NaCl/0.05M Tris, pH7.0/0.001M EDTA/0.5% SDS) was used to equilibrate the oligo(dT) to pH7.25.

Prior to chromatography separation, RNA was dissolved in 500µl of a solution containing 10mM VRC (Vanadyl Ribonuclease complex)/50mM Tris, pH7.0/0.5% SDS and 0.4M NaCl. The columns weres loaded with 100µl of tRNA (10mg/ml). The tRNA was eluted with 10 volumes of Bb$^+$. The RNA samples were then applied and the eluates collected and reapplied four times to maximize poly(A)$^+$ mRNA binding. Six column volumes of Bb$^+$ was used to elute the poly(A)$^+$ mRNA. Final eluants were collected and precipitated with 2M NH_4OAc and 2.5 volumes 95% EtOH overnight at -20^{0}C.

The poly(A)$^+$ mRNA pellet was washed and dissolved in DEPC.ddH_2O and its concentration and integrity determined as outlined above prior to storage at -70^{0}C (see Newt RNA Isolation).

In Vitro Translation of Isolated RNA

The rabbit reticulocyte lysate purified of any endogenous mRNA was used to translate isolated poly(A)$^+$ mRNA. Two microlitres of mRNA (0.5ug/ml) were mixed on ice with 35µl of nuclease-treated lysate, 7µl ddH_2O, 1µl amino acid mixture minus leucine (1mM) and 5µl ^{3}H-leucine (sp. act.=100-200Ci/mmole).

The reaction was allowed to proceed for 60 min at 30^{0}C with 5µl aliquots being removed at time intervals (t=0 min, 10 min and 30 min) and assayed for ^{3}H-leucine incorporation (see TCA Protein Precipitation). Upon completion of the *in vitro* translation reaction, a further TCA protein precipitation was done to determine ^{3}H-leucine incorporation prior to the addition of equal counts per minute (cpm) for gel electrophoretic analysis of the protein translation products (see Flurography).

Transformation of *Escherichia coli* by plasmid DNA

Plasmid transformation was performed as outlined in Maniatis *et.al.* (1982). Fifty millilitres of L broth (LB) was inoculated with 0.5ml of an overnight culture of host JM109 [recA$^-$, hsdR17, W(lac-proAB)] bacterial cells grown at 37^{0}C in a shaking incubator. The culture was grown at 37^{0}C for 2-4 h until a concentration of approximately 5 x 10^{6}cells/ml was achieved [N.B. for recA$^-$ strains, i.e. JM109, 1 $O.D._{600}$ = 0.5(5 x 10^{6}cells/ml)]. At this time the culture was chilled on ice for 10 min and then centrifuged for 10 min at 2500rpm (4^{0}C) in an IEC Centra-7R centrifuge. The pellet was resuspended in fresh, ice-cold 50mM $CaCl_2$/10mM Tris, pH8.0 (0.5 vol) and left on ice for 30 min. After centrifuging the cells were gently resuspended in 0.1 volume of 50mM $CaCl_2$/10mM Tris-HCl, pH8.0.

A maximum of 40ng of plasmid DNA (e.g. pMHS-243) was added to 200µl of cells per transformation and left on ice for 1-2 h. 100ul of the

transformed cells were spread on L broth + ampicillin (40µg/ml) plates and left overnight at 37^{0}C.

Antibiotic-resistance colonies were picked and used in rapid and large scale plasmid preparations.

Rapid Plasmid Preparations, (Birnboim and Doly, 1979)

From 5ml overnight antibiotic cultures, 1.5ml was placed into an Eppendorf tube and spun for 1 min in a Fisher microfuge. The pellet was resuspended in 100µl of fresh, ice-cold 50mM glucose/10mM EDTA/25mM Tris, pH8.0 + 4mg/ml lysozyme and left at room temperature for 5 min. Two-hundred microlitres of ice-cold 0.2N NaOH/1% SDS were added and the Eppendorf vortexed and left for 5 min on ice. Ice-cold 5M potassium acetate, pH4.8 (150µl) was added and the solution vortexed and left on ice for another 5 min. The supernatant was transferred to a new Eppendorf after a 5 min spin at 4^{0}C and extracted with phenol:chloroform:isoamyl alcohol (P:C:IA, 25:24:1). Two volumes of room temperature absolute ethanol were mixed with the aqueous phase and allowed to sit for 2 min prior to centrifugation (5 min) at room temperature. The DNA pellet was washed in 70% ethanol and dried under vacuum for 5 min. The pellet was resuspended in 50µl of 1xTE, pH7.2 + 50µg/ml RNase A. Approximately 0.5µg plasmid DNA (as determined by spectrophotometrically) was used for each restriction enzyme digestion to test for the cloned insert integrity.

Large-Scale Isolation of Plasmid DNA

From overnight 5ml cultures (37^{0}C) set up in LB with appropriate antibiotic, 0.1ml was inoculated into 25ml LB medium + antibiotic in a shaking 37^{0}C incubator. The culture was grown until it reached late log phase ($O.D._{600}$ = 0.6) then added to 500ml of LB medium + antibiotic

(pre-warmed to 37^{0}C) in a 2L flask. Exactly 2.5 h after incubation in a shaking 37^{0}C water-bath, chloramphenicol was added to a final concentration of 170µg/ml and the culture incubated for a further 12-16 h to allow for plasmid amplification.

Bacterial cells were pelleted (5000rpm, 10 min, 4^{0}C) in an IEC PR-600 centrifuge and resuspended and washed in 0.1-0.2 volumes of ice-cold STE. The suspension was again spun and the bacterial pellet was resuspended in 50mM glucose/25mM Tris, pH8.0/10mM EDTA solution containing 5mg/ml lysozyme. The suspension was transferred to a polypropylene centrifuge tube and left at room temperature for 5 min. Twenty millilitres of freshly made 0.2N NaOH/1% SDS solution was added and the tube was mixed by inversion and left on ice for 10 min. A further 15ml of ice-cold 3M potassium acetate/1.8M formic acid solution was added and the tube was vortexed prior to being cooled on ice for 20 min.

The supernatant was collected (15000g, 15 min, 0^{0}C) and transferred to fresh tubes containing 0.6 volumes of isopropanol. The solution was mixed by inversion and the nucleic acid precipitate formed after 15 min at room temperature. The supernatant was discarded after centrifugation (15000g, 30 min, 0^{0}C) and the DNA pellet washed in 70% ethanol and dried *in vacuo* for 5 min prior to being dissolved in 1xTE, pH7.2 (10ml) and transferred to Beckman ultrafuge tubes.

The exact volume of the aqueous DNA solution was measured and 1g of $CsCl_2$ was added and dissolved per ml of solution. Ethidium bromide was added to the $CsCl_2$ solution at a final concentration of 0.1mg/ml and the balanced tubes were sealed with a Beckman Tube Topper. The tubes were loaded into an IEC A-321 head and centrifuged at 45000rpm for 36 h at 20^{0}C.

The recombinant plasmid DNA band was visualized under UV light, below the chromosomal DNA band. Removal of the plasmid band into corex tubes was accomplished by using a 20 gauge needle attached to a 3ml syringe.

The ethidium bromide was removed by several extractions with water-saturated 1-butanol. The aqueous phase was diluted with 3 volumes of ddH_2O to which 0.3 volumes of 3M NaOAc had been added. The plasmid DNA was precipitated with 3 volumes of 95% ethanol overnight at -20^0C. The plasmid pellet was collected (10000rpm, 30 min) and washed with 70% ethanol, dried and dissolved in1xTE, pH7.2 (1ml).

An optical density reading was then taken on a Beckman DU-7 spectrophotometre in order to elucidate the concentration and purity of the plasmid DNA. An O.D. of 1.0 at 260nm is roughly equivalent to 50µg/ml of d.s. DNA (Maniatis *et.al.*, 1982) while the purity was determined by the 260nm:280nm ratio. A ratio of 2:1 suggests little protein contamination. All DNA samples were stored at 4^0C or at -20^0C for longer periods.

Nick Translation

In an Eppendorf 0.5µl of the three non-labelled dNTPs (dTTP, dGTP and dATP) each 10mM were added to 2.5µl of 10xNTB, pH7.2 and 13µl DEPC.ddH_2O. One microlitre containing 0.25µg of restriction enzyme digested and electro-eluted insert cDNA, pMHS-243, was added to the mixture and put on ice. A DNase I stock solution (1mg/ml) was diluted 1000 fold and put on ice. Behind a plexiglass shield, 5µl of α^{32}P-dCTP (sp. act.=800Ci/mmol) was added along with 1µl of the diluted DNase I solution and 1µl of DNA polymerase I (conc.=5U/µl). The reaction was allowed to proceed for 1.5 h in a 14^0C water-bath. The reaction was stopped by the addition of 100µl of stop buffer (50mM EDTA/0.1mg/ml Bovine serum albumin [BSA]) and placed on

ice ready for spun-column separation of the radiolabelled probe from unincorporated hot and cold nucleotides.

Oligolabelling by Random Primers

To an Eppendorf containing 2µl (25ng) of purified denatured (90^0C, 15 min) restriction digested pMHS-243 cDNA was combined 24.5µl ddH_20, 5µl reagent mix (dATP, dGTP, dTTP and $p[dN]_6$), 1µl BSA, 2.5µl (50µCi) α^{32}P- dCTP and 1µl Klenow (conc.=5U/µl). The reaction was allowed to proceed overnight at room temperature behind plexiglass and terminated by the addition of 100µl of stop buffer. The cDNA probes were purified by spun-column separation.

Spun-Column Separation

This procedure was followed as outlined by Maniatis *et.al* (1982).

Sephadex G-50 in STE, pH8.0 was packed into a disposable, 1ml tuberculin syringe plugged with sterile glass wool by spinning in a table top centrifuge (1700rpm, 5 min). The column was rinsed several times in STE (1700rpm, 5 min) prior to the addition of the radiolabelled probe (125µl) to the column.

The column with the DNA sample was spun at 1700rpm for 5 min and the eluate containing the radiolabelled DNA was collected in an Eppendorf tube. The incorporation of α^{32}P dCTP into the probe was measured using a Beckman LS 1800 scintillation counter and 10ml aqueous counting scintilant (Amersham). Most preparations had a specific activity greater than 3 x 10^8cpm/µg. The probe could now be stored (-20^0C) or be denatured in boiling water for 10 min prior to the addition to the hybridization solution.

Southern Transfers

Restriction digested genomic DNA samples incubated in 6x loading buffer were electophoresed in a 0.8% agarose gel prepared in 1xTBE and

running buffer. The gel was stained in ethidium bromide and visualized under a UV light source.

The DNA was acid nicked by placing the gel in 0.25N HCl at room temperature for 10 min with constant agitation. The gel was then rinsed in dH_20 before a 10 min neutralization in 0.4N NaOH. The DNA was adhered to side B of a pre-treated Gene Screen Plus membrane (soaked 10 min in10xSSC) by overnight capillary transfer through 10xSSC (outlined in Gene Screen Plus Manual, 1985). The next day the filter was allowed to air dry face up (side B) on Whatman prior to low stringency hybridization.

A low stringency hybridization condition allowing for approximately 25% nucleotide mismatching (Moran *et.al*, 1983; Lowe and Moran, 1986; Gonzalez *et.al*, 1987) was achieved by modification of Gene Screen's stringency protocol. The filters were placed into a polyethylene bag and sealed on 3 sides with an Impulse Sealer (TEW). The low stringency hybridization solution (10ml), consisting of 40% formamide/8% sodium dextran/0.85% SDS/1.2M NaCl and 0.4mg/ml denatured carrier herring sperm DNA was added to the bag. All bubbles were expelled before sealing the final side. The filter in the hybridization solution was placed in a 37^0C shaker water-bath and held flat with weights around its periphery for a minimum of 4 h.

Upon completion of the prehybridization, the previously denatured radiolabelled probe (see Nick Translation or Oligolabelling by Random Primers) was added at a specific activity of 1×10^7cpm/ml to the hybridization solution. Bubbles were expelled and the bag was sealed and checked for leaks. The re-sealed bag was then incubated at 37^0C for approximately 72 h.

The hybridized filters were drained of the hybridization solution and washed two times for 15 min with 250ml of 2xSSC at room temperature. The

filter was then washed two times for 30 min in 500ml of 2xSSC/1.0% SDS in a 50^{0}C shaking water-bath. Non-specific radioactive background was checked with a hand held Geiger counter and further washes were done at 60^{0}C if needed.

Upon completion of the washes, the Gene Screen filter was blotted and sealed wet in a polyethylene bag. The hybridized filter was sandwiched between two Kodak X-Omat AR films exposed in a Wolf cassette containing Cronex intensifying screens. The cassette was placed at -80^{0}C for a minimum of 48 h before developing.

The films were developed in Kodak GBX Developer and Replenisher at 20^{0}C for 5 min. The films were then rinsed in a water stop bath for 30 sec and fixed at 20^{0}C for 4 min in Kodak GBX Fixer and Replenisher. The exposed films were again washed in a water-bath for 5 min and then allowed to dry.

Electrophoresis of Glyoxalated RNA (Northern)

Lyophilized RNA (20µg) was dissolved in 5µl of 6M glyoxal (neutralized by mixed bed resin, Bio-Rad AG 501-X8), 3µl of 100mM sodium phosphate and 22µl of DEPC.dd$H_2$0. The solution was incubated at 50^{0}C for 30-60 min and cooled on ice prior to the addition of 6x loading buffer.

The denatured RNA solution was loaded into a 1.2% agarose gel composed of 10mM sodium phosphate, pH6.8 and run at 80V for the required time period. The 10mM sodium phosphate, pH6.8 running buffer was recirculated throughout the duration of electrophoresis to maintain the pH below 8.0.

Upon completion of electrophoresis, the gel was stained with ethidium bromide to check the relative amounts of sample loaded as well as the integrity of the RNA. The RNA was transfered to side B of pre-treated Gene Screen Plus (soaked 10 min in 10xSSC) and transferred through 10xSSC

overnight. The next day the filter was removed from the gel and soaked sequentially in a solution of 50mM NaOH for 30 sec and in a solution of 0.2M Tris, pH7.6/2xSSC for 30 sec.

The membrane was allowed to dry face up (side B) prior to low stringency hybridization of the radiolabelled probe (see Southern Transfer).

Probes and Antibodies

A murine hsp70-like probe was a generous gift of Dr. R. Kothary of Mt. Sinai Research Institute of Toronto. The cDNA probe, pMHS-243, was blunt end ligated into the plasmid pEMBL8 (see Appendix). *In vitro* translation of the open reading frame (420 codons) encodes for a protein with the same mobility as the heat inducible hsp68 (Low and Moran, 1986). Comparison of pMHS-243 predicted amino acid sequence to *Xenopus*, *Drosophila* and yeast hsp70 revealed homology of 81%, 74% and 76%, respectively (Low and Moran, 1986). Northern blot analysis of mouse control and heat shock RNAs using the cDNA insert of pMHS-243 indicated intense hybridization to the induced hsp68 mRNA and the constitutively expressed hsc74 and hsc70 transcripts. The cross-reaction to the cognates was due to the long 5' untranslated sequences contained in this cDNA (Low and Moran, 1986).

A *Xenopus* hsp70 genomic probe (2.2Kb) containing 5' promoter sequences was a generous gift of Dr. M. Bienz of MCR Laboratory, Cambridge. The insert was cloned into an Xho I restriction site of the plasmid pUC12 (see Appendix). The genomic fragment encoded for a single open reading frame protein of 647 amino acids that had 74% homology to the *Drosophila* inducible hsp70 (Bienz, 1984a).

Two mouse monoclonal antibodies prepared against the human inducible (hsp72) and constitutive + inducible (hsp72 and hsc73) forms of HSP70 were a generous gift of Dr. W. Welch of Cold Spring Harbor

Laboratory. These antibodies designated C92 (hsp72) and N27 (hsc73 + hsp72) were used in Western blot analyses (Welch and Feramisco, 1984).

A mouse monoclonal antibody (Ub1) prepared against the human ubiquitin protein was a generous gift of Dr. S. Showalter of the National Cancer Institute, Maryland.

Results

Induced Protein Levels Are Not Due to Benzocaine

Since the HSP promoters are responsive to a number of stress stimuli including heat shock (see Table II), it seemed possible that the electrophoretic pattern of the proteins extracted from the newt forelimb may be due to the potential stress of the anesthetic, ethyl-methylbenzoate, and not to the trauma of amputation. To address this possibility, protein was extracted from unamputated, untreated and benzocaine-treated newts respectively, separated by SDS-gel electrophoresis and stained with Coomassie blue (Figure VIA). Newts were treated with benzocaine for either 15 min (normal control time period) or 1.5 h (to assess its' possible residual influence). Comparisons of patterns of stained proteins from limbs and tails of the corresponding non-anesthetized and anesthetized animals on gels revealed no prominent differences in the profiles of the larger molecular weight proteins. Slight differences were occasionally evident in the lower molecular weight proteins (less than 20kDa) though poor resolution and separation hindered their further analysis. Additionally, no differences in protein compositions were observed in the 1.5 h tail and limb lanes that could be attributed to residual effects of ethyl-methylbenzoate.

Further analysis of these stained gels by scanning densitometry (data not shown), verified a lack of apparent differences (quantitative or qualitative) in the higher molecular weight proteins between anesthetized and non-anesthetized newts. Ethyl-methylbenzoate was also observed to have little effect on the proteins synthesized systemically as judged by a comparison of polypeptides extracted from the tail (T) and limb (L) of individual unamputated newts. It seems, therefore, that any change in protein pattern resulting from amputation should reflect the trauma of

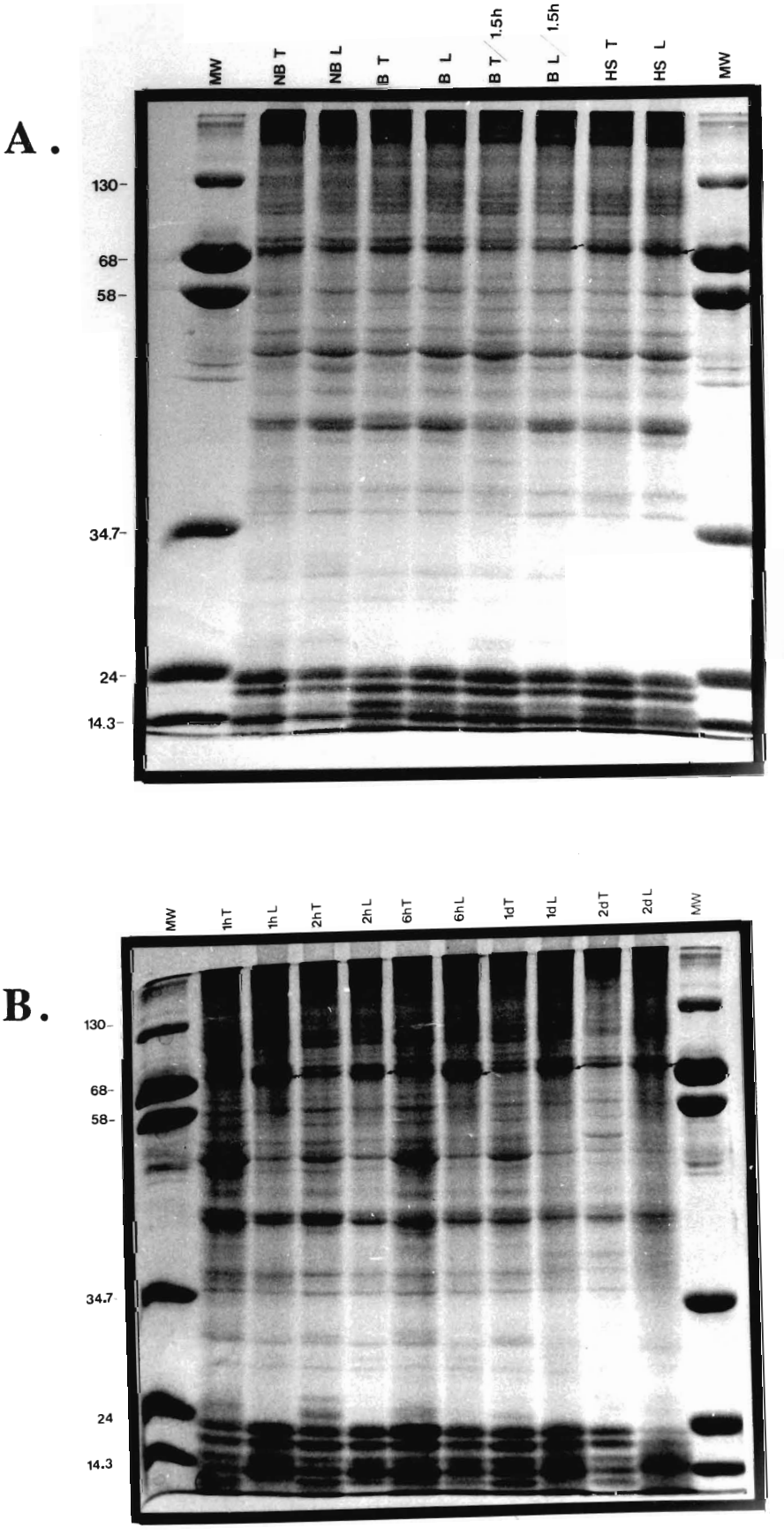
Figure VI. Coomassie blue stained 10% SDS-PAGE of total (70µg) soluble proteins. (A) represents protein patterns of non-anesthetized and anesthetized unamputated newts. (B) represents protein isolated at various time intervals post-forelimb amputation (p.f.a.) of anesthetized newts. Molecular weight markers (kDa) corresponding in descending mass are ß-galactosidase, BSA, catalase, pepsin, trypsinogen and lysozyme.

A) NB T= non-benzocained tail tissue
NB L= non-benzocained limb tissue
B T= benzocained (15 min) tail tissue
B L= benzocained (15 min) limb tissue
B T/1.5 h= benzocained (1.5 h) tail tissue
B L/1.5 h= benzocained (1.5 h) limb tissue
HS= heat shock (31^0C for 1 h)

B) 1 h T= 1 h tail tissue (p.f.a.)
1 h L= 1 h distal limb stump (p.f.a.)
2 h T= 2 h tail tissue (p.f.a.)
2 h L= 2 h distal limb stump (p.f.a.)
6 h T= 6 h tail tissue (p.f.a.)
6 h L= 6 h distal limb stump (p.f.a.)
1 d T= 1 day tail tissue (p.f.a.)
1 d L= 1 day distal limb stump (p.f.a.)
2 d T= 2 day tail tissue (p.f.a.)
2 d L= 2 day distal limb stump (p.f.a.)

Arrows indicate accumulation of 70kDa limb protein.

Figure VI. Coomassie Blue Stained SDS-PAGE



amputation or heat shock and not the effects of ethyl-methylbenzoate. Therefore in all subsequent experiments, newts were treated with benzocaine prior to all surgical manipulations. In addition, it was demonstrated that the protein spectrum, on one-dimensional SDS-gels, of the tail and forelimb extracts of unamputated newts revealed no major differences. Hence unamputated forelimbs, representing a natural control, were used in comparison to limbs of surgically manipulated newts.

Forelimb Amputation Induces Localized and Systemic Changes in Protein Levels

Relative to the unamputated control forelimbs, qualitative and quantitative differences in protein levels were observed in the extracts derived from distal forelimb stump tissue of amputated newts at various times post-amputation (Figure VIB). The most obvious change occurred in a protein with an approximate molecular weight of 70,000 daltons and was observed at all times subsequent to amputation. High levels of the 70kDa protein are observed as early as 1 h post-amputation and are maintained for at least a period of 6 h prior to their gradual decline at day two post-amputation.

In corresponding tail extracts of animals with amputated forelimbs, this 70kDa protein appears to be of approximate control levels. This would indicate a non-systemic 70kDa protein change that is induced by mechanical amputation of the forelimb.

Forelimb amputation has effects on other proteins present in the distal stump and tail tissues. Densitometry of gels containing stained protein isolated from distal limb stump and tail sections one hour after forelimb surgery allows the identification of a number of differences and similarities relative to the unamputated control limb (Figure VII). Comparison of the

aligned densitometry scans reveals that the presence or repression of many early wound response proteins become specific for their locale (i.e. tail relative to distal limb stump) after the mechanical trauma of limb amputation. For example, the protein peak designated 'b' (Figure VII), in the 1 h tail extraction appears to be independent in its accumulation with respect to peak 'b' of the 1 h limb and unamputated control samples. Interestingly, alignment of the scans suggest that the 70kDa protein, designated 'a', may actually be synthesized *de novo* upon amputation.

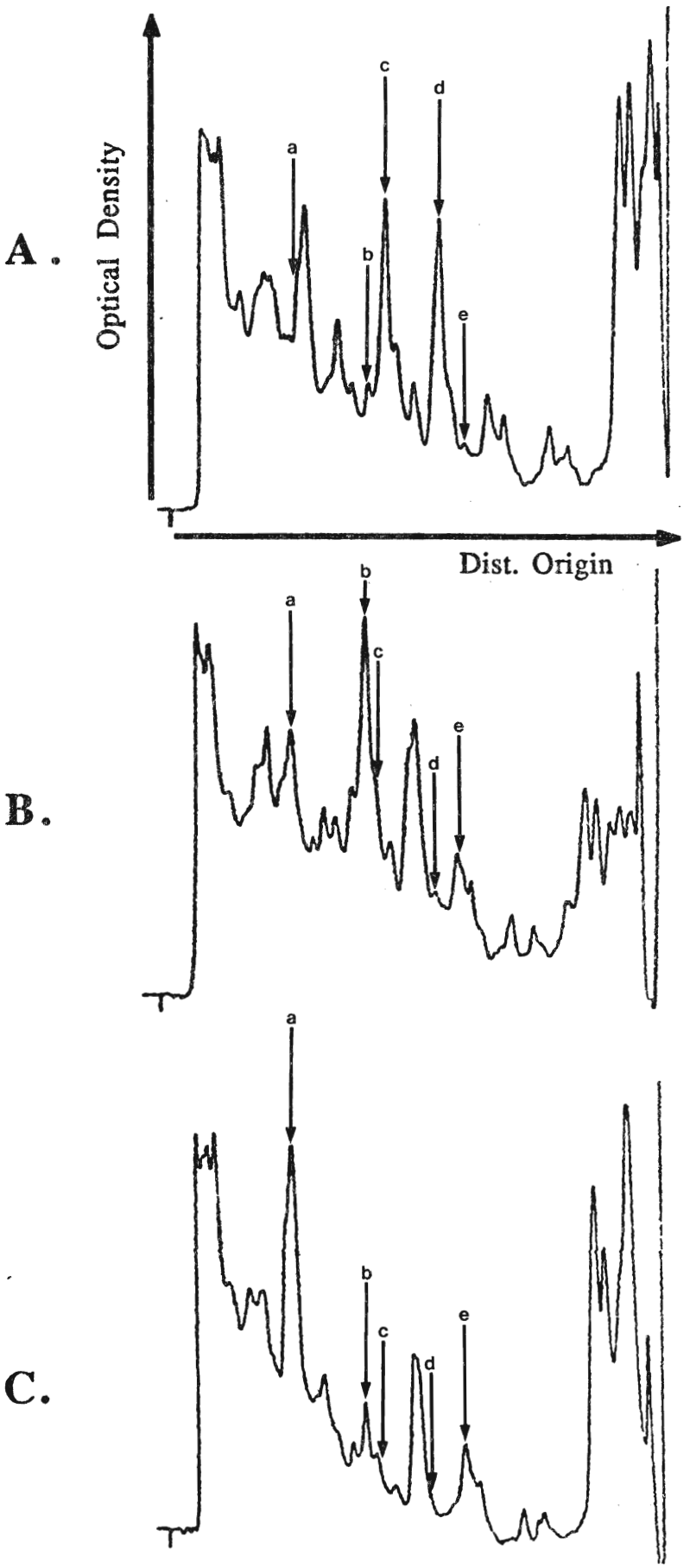
Evidence for the apparent restoration of the protein spectrum (equivalent to that of the control unamputated limb) in the tail and limb, by day 2, was provided by densitometric scans of Figure VIB. Restoration of the protein spectrum in the distal stump tissue of amputated forelimbs was coordinated with the decline of the 70kDa protein (data not shown). Since similar protein patterns were present in replicate gels it is unlikely that these results were due to intrinsic experimental artefacts or within-animal variance.

Effects of Heat Shock on Electrophoretic Pattern of Coomassie Stained Protein

Preliminary experiments in which whole newts received a mild heat shock (31^0C for 1 h) showed a slight increase in the accumulation of a 70kDa protein; the presumptive hsp70 (Figure VIA). This result is in agreement with that of Rutledge *et.al.* (1987) who found that a mild heat shock for 1 h at 5^0C below CTM resulted in minimal induction of the HSPs of the 70kDa family in tissues of the salamander, *Eurycea bislineata* This initial heat shock protein electrophoretic profile was in many cases similar to that of protein extracted from newt forelimb stump tissue at 1 h post-amputation (Figure VIA/B).

Figure VII. Densitometre scans corresponding to the protein patterns from Figure VI. (A) is the representative pattern of the control (Cont.) unamputated forelimb from anesthetized newts. (B) is the representative pattern of the early systemic tail (1 h T) p.f.a. response. (C) is the representative pattern of the early localized distal limb stump (1 h L) p.f.a. response. Alignment of lower case letters (i.e. a-e) indicate some higher molecular weight protein differences relative to the control.

Figure VII. Densitometre Scans of Coomassie Stained SDS-PAGE



The Trauma of Forelimb Amputation Mimics the HSP Response

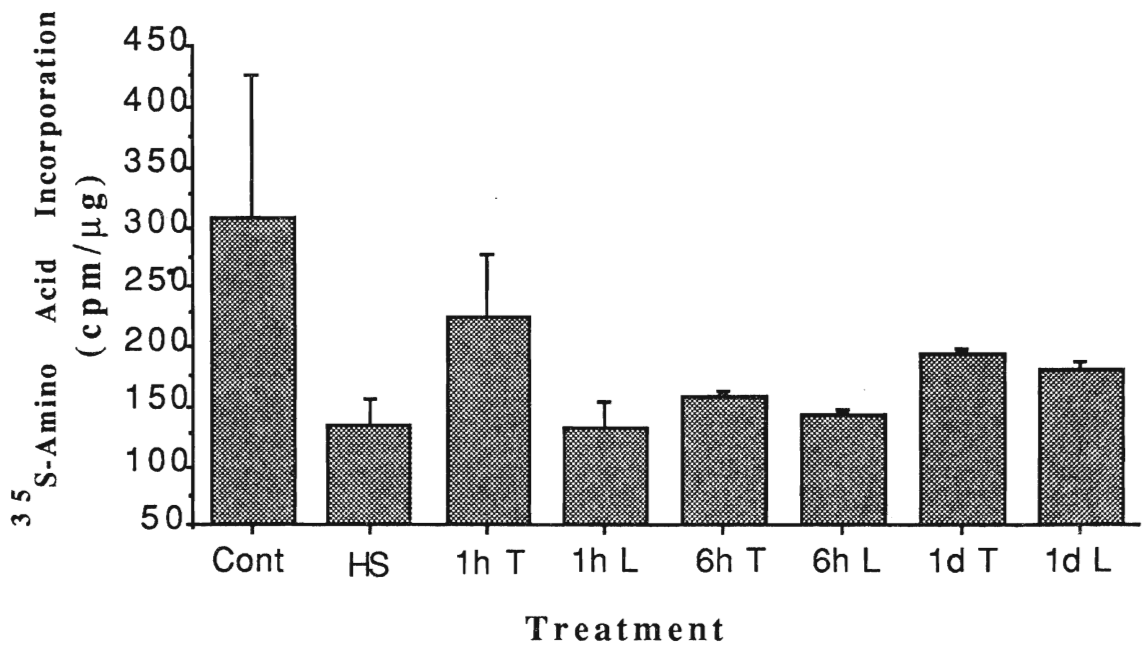
Most species respond to extreme alterations in their natural environment by altering their repertoire of proteins synthesized. Such a changeover in the proteins being synthesized is generally reversible, since upon removal of the stress condition, restoration of normal protein synthetic patterns is observed. The results shown in Figures VIA and VIB, represent electrophoretic patterns of Coomassie stained total soluble proteins present in specific tissues and do not indicate relative or absolute values of protein synthesis. Newly synthesized proteins can be visualized by fluorography after pulse labelling with radioactive amino acids.

^{35}S-labelled amino acid incorporation into total TCA-insoluble protein prior to and subsequent to amputation or heat shock are shown in Figure VIII. This figure illustrates the transient changes in the absolute value of protein synthesis. Extracts prepared from amputated newt forelimbs show a strong decrease in the incorporation of labelled methionine/cysteine compared to unamputated control limbs up to 24 hours after surgery. This overall decrease in protein synthesis is characteristic of the heat shock response. Easton *et.al.* (1987) and Rutledge *et.al.* (1987) observed a decline in ^{35}S-incorporation, in heat shocked salamanders, of approximately 11 h to 24 h before restoration to the basal control value occurred. The kinetic time course of recovery from heat shock, as demonstrated by Easton *et.al.* (1987) and Rutledge *et.al.* (1987), was comparable to that measured for the restoration of control protein synthetic rates seen after limb amputation. Thus, Figure VIII demonstrates that the time course for recovery of protein synthesis in forelimbs in response to amputation is less than 24 h and not 48 h as previously suggested by results from the Coomassie blue stained SDS-PAGE (Figure VIB).

Figure VIII. Bar graph of absolute TCA-insoluble protein fractions isolated from 4 h radiolabelled tissue homogenates.

Cont.= unamputated forelimb
HS= heat shock (34.5^{0}C)
1 h T= 1 h tail (p.f.a.)
1 h L= 1 h distal limb stump (p.f.a.)
6 h T= 6 h tail (p.f.a.)
6 h L= 6 h distal limb stump (p.f.a.)
1 d T= 1 day tail (p.f.a.)
1 d L= 1 day distal limb stump (p.f.a.)

Figure VIII. Absolute Protein Synthesis



When individual newts were heat shocked at 2^0C below their CTM (36^0C), a rapid repression of normal cellular protein synthesis was also observed (Figure VIII). This value, indicating rapid repression of total protein synthesis, was comparable to the 1 h post-amputated forelimb value but is considerably lower than values obtained for 1 h tail and the forelimb and tail extracts obtained at later times post limb amputation. Interestingly, no significant systemic effect of forelimb amputation was observed in tail tissues up through 6 hours post-amputation.

Qualitative Comparisons of Protein Synthesis During Heat Shock and After Forelimb Amputation

Heat shock proteins are defined as proteins whose relative synthesis is enhanced in response to the stress of hyperthermia. A qualitative analysis of the patterns of newly synthesized proteins by SDS-PAGE and fluorography was performed. Gels (Figure IXA/B) were loaded with either equal counts (10,000cpm) or protein (75µg), respectively. (A) compares the relative changes in the levels of individual proteins synthesized while (B) compares the changes in the total levels of protein synthesis due to heat shock (34^0C/1 h) and amputation (1 h post-amputation). This qualitative and quantitative pattern of the protein array was reproducible in duplicate experiments.

Figure IXA, demonstrates that the mechanical injury of forelimb amputation was sufficient to induce qualitative and quantitative changes in protein synthesis analogous to those induced by hyperthermia. Amputation induced the synthesis of two proteins whose molecular weights were similar to the hsp70 and hsp30 proteins synthesized in response to a 1 h heat shock at 34^0C. The 70kDa protein synthesized in response to amputation is found in

distal limb stump tissues but is not seen in tissues removed simultaneously from the tail. The synthesis of the 30kDa protein on the other hand, appears to be induced systemically in response to limb amputation.

Synthesis of the 70kDa protein in response to amputation reflects the accumulation of Coomassie blue stained protein in that size range seen previously (Figure VIB). In contrast, resolution of low molecular weight proteins on the fluorographs was poor compared to Coomassie blue stained gels. The use of tritiated amino acid residues would help to solve this problem as Welch (1985) observed that smaller mammalian stress proteins do not effectively label with ^{35}S-methionine.

Unlike the other protein spectra of Figure IXA, the tail showed a markedly increased rate of synthesis of a protein located at approximately 46kDa. This protein, as described by Rutledge (1987), was most likely newly synthesized actin.

As can be seen in Figure IXB, the pattern of protein synthesis in forelimb stump tissue after amputation mimics that in response to heat shock. That is to say, there is a general repression of total protein synthesis and the specific increased synthesis of a 70kDa and 30kDa protein. The limb amputation leads to a marginal inhibition of total protein synthesis in the tail.

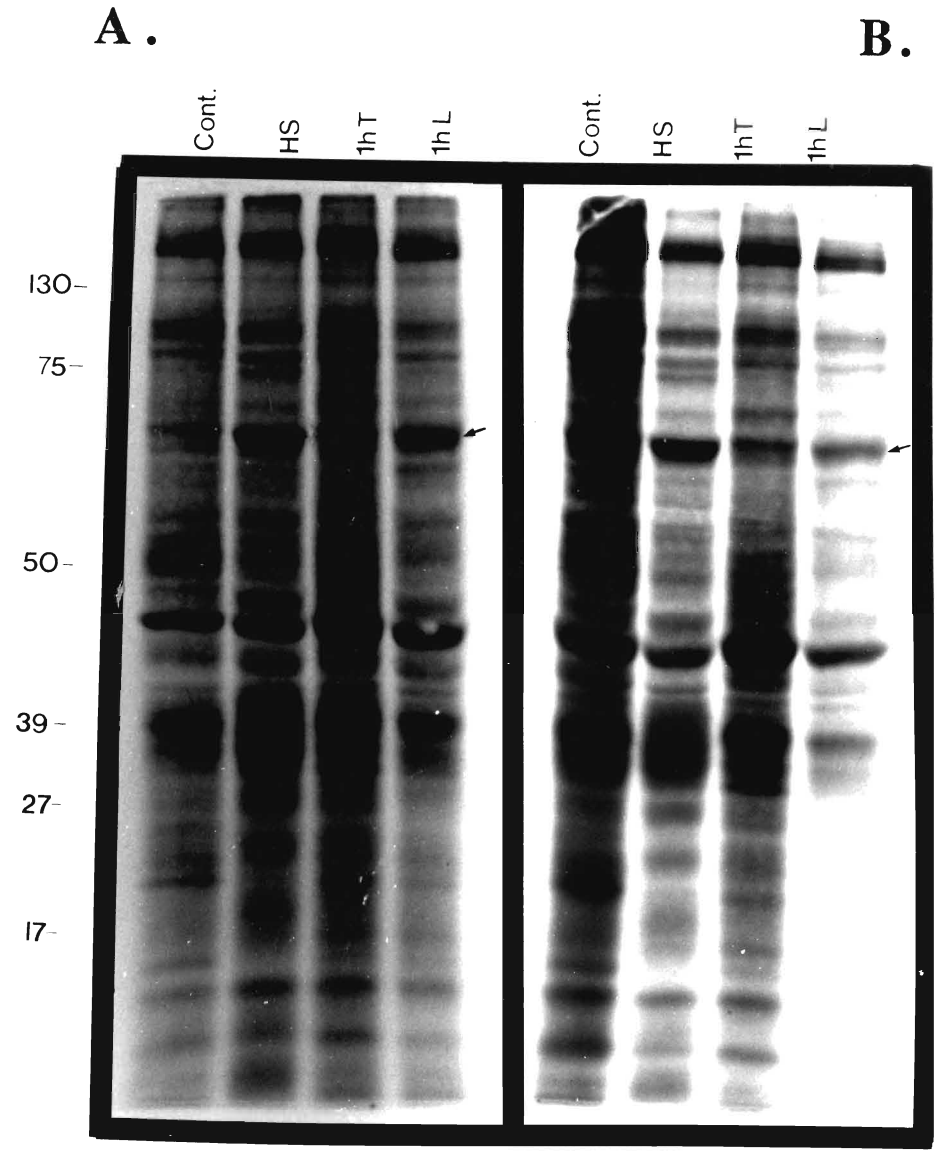
Overall protein synthesis rates are generally depressed following a stress response severe enough to eventually induce hsp or hsp-like synthesis. Thus examination of fluorograms, or even densitometric data in the form of hsp synthesis can sometimes be misleading. To control for this, equal cpm (relative synthesis) should be electrophoretically run and if possible proteins should be normalized against other proteins of similar molecular weights that do not change during experimental conditions

Figure IX. Fluorograms (20 day exposure) of 10% SDS-PAGE showing the effects of a one hour heat shock and amputation through the humerus on protein synthesis in the forelimbs of the adult newt. (A) 10,000cpm of ^{35}S-methionine radiolabelled protein was loaded per lane; indicates relative synthesis. (B) 75µg of ^{35}S-methionine radiolabelled protein was loaded per lane; indicates absolute synthesis. Molecular weight markers in descending mass are given in kilodaltons.

Cont.= unamputated forelimb
HS= heat shock (34.5^{0}C)
1 h T= 1 h tail (p.f.a.)
1 h L= 1 h distal limb stump (p.f.a.)

Arrows indicate accumulation of a 70kDa protein in the 1 h distal limb stump (p.f.a.).

Figure IX. SDS-PAGE Fluorograms



(Colbert and Young, 1987). Rutledge *et.al.* (1987) calculated relative rates ofprotein synthesis by multiplying percent of total protein synthesis by the overall protein synthesis, determined by TCA-incorporation of methionine. For the these reasons, the relative peak abundance for Figure IXB (absolute values) will not be discussed but is graphically represented in the Appendix.

Figure X summarizes the relative levels of induction of the major proteins characterized in Figure IXA. Each fluorogram lane was scanned by a densitometre and the area of each radiolabelled protein band, derived from planimetre tracings, was expressed as percent relative abundance in comparison to the total integrated area per lane. These data demonstrate that the most dramatic change in protein synthesis, in the heat shocked newts and the 1 h distal limb stump, occurred in proteins of MW 70kDa (an increase from 5% to 11% of total labelled protein) and 30kDa (an increase from approximately 5% to 17% of total labelled protein). Ratios of total areas normalized to the control indicate a minimum increase of 200% and 150% for the 70kDa and 30kDa protein, respectively, due to heat shock and amputation (1 h L). In note, the relative synthesis of the amputation induced 70kDa and 30kDa proteins may be an underestimation due to decreased radiolabel incorporation time subsequent to and after amputation.

Qualitative Analysis of Protein Synthesis After Heat Shock and Forelimb Amputation by Two-Dimensional Gel Fluorography

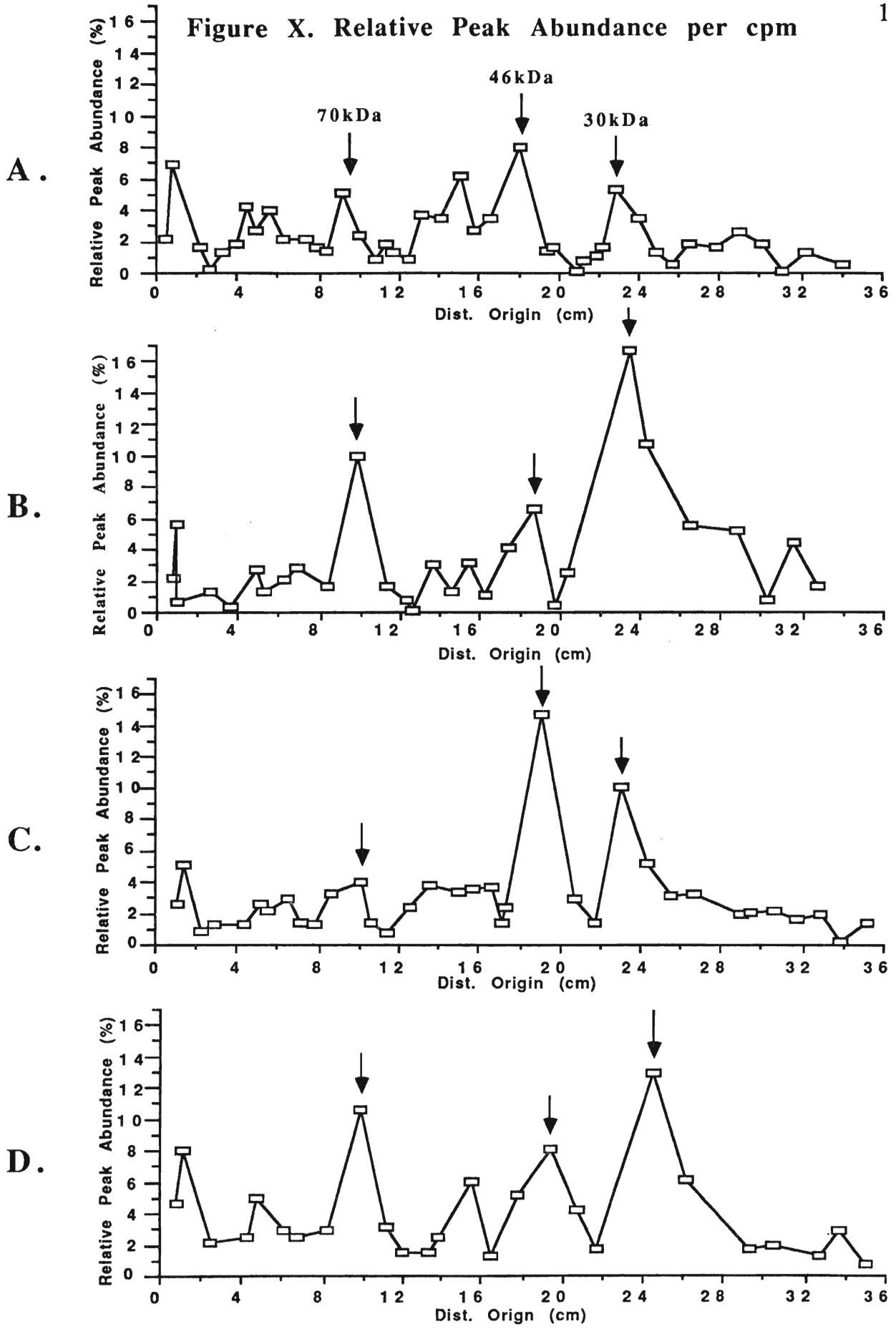
Figure XI depicts a detailed analysis of relative protein synthesis patterns in newt forelimbs by two-dimensional IEF-PAGE fluorography. Each first dimensional IEF gel is loaded with equal counts of radiolabelled protein (Coomassie blue stained 1D IEF gels and corresponding 2D silver stained gels appear in the Appendix). In Figure XIB, one observes both dramatic and subtle differences between the array of proteins synthesized in the limb in

Figure X. Graphical representation, corresponding to Figure IXA, of the relative level of ^{35}S-methionine incorporation into the TCA-insoluble protein fraction. Protein peaks are expressed as a percentage of total integrated area.

(A) unamputated control forelimb
(B) heat shock
(C) 1 h tail (p.f.a.)
(D) 1 h distal limb stump (p.f.a.)

Proteins corresponding to 70kDa, 46kDa and 30kDa are indicated by arrows.

Figure X. Relative Peak Abundance per cpm



response to heat when compared to those found in the control (A) and from tail and limb tissue (C and D) isolated from forelimb amputated newts. The characteristic comigration of the highly inducible hsp70 isoforms and hsc70 cognate protein is in agreement with their human analogues, hsp72 and hsc73, (Chappell *et.al*, 1986). These hsp70 (pI=6.5-6.8) proteins were only prominent in the heat shocked newts (B). The cognate hsc70 form (being slightly more acidic than hsp70) was discernible in all the fluorograms with the exception of the 1 h post-amputation forelimb (D). There appears to be a low level of synthesis of a complex array of low molecular weight hsps in (B) that share some homology with those found in the limb at 1 h post-amputation (C). Resolution of these small stress proteins is poor. Tritiated protein samples were sent to Dr. W. Welch (Cold Spring Harbour) for analysis, but no better resolution was obtained.

The most significant change observed at 1 h post-amputation (Figure XID), was the appearance of 70kDa protein in the limb with a more acidic pI. This protein, which has been termed amp70, can be observed at low levels in the control limb and tail homogenates (A and C, respectively). It appears to be synthesized negligibly, if at all, in the limbs of heat shocked newts (B). The identity of this amputation-induced forelimb protein remains to be determined.

Accumulation of Amp70 and Inducible Hsp70 Correlates With Increased HSP70 mRNA Transcripts

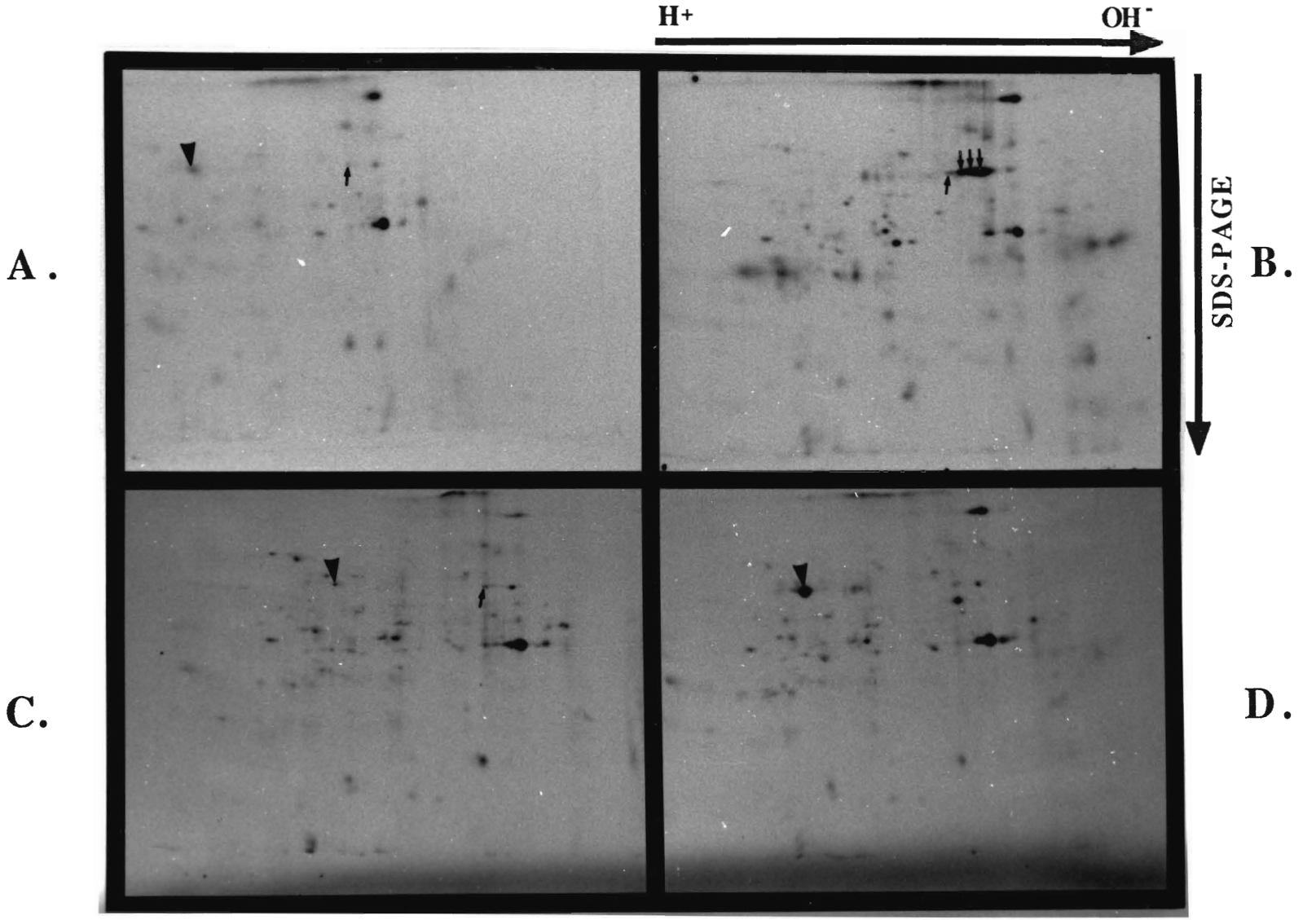
To determine if the increase in synthesis of amp70 seen in response to amputation was correlated with an increase in hsp70 mRNA synthesis, Northern hybridization was performed. Hybridization of a radiolabelled HSP70 probe to mRNA isolated from an amputated newt would enable the characterization of the transcript size(s) and its level of expression relative

Figure XI. Fluorograms (20 day exposure) of two-dimensional polyacrylamide gels (10%) showing the effects of one hour heat shock and bilateral amputation (through the humerus) on protein synthesis in the forelimbs of the adult newt. 5,000cpm were loaded per IEF (3.5-10/6-8 ampholytes) gel.

(A) unamputated control limb
(B) heat shock (34.5^{0}C)
(C) 1 h tail (p.f.a.)
(D) 1 h distal limb stump (p.f.a.)

The large arrowheads in (A), (C) and (D) point to the 70kDa protein induced by amputation (i.e. amp70). The downward arrows in (B) point to the inducible hsp70 proteins while the upward arrows in (A), (B) and (C) point to the constitutive hsc70 protein.

Figure XI. Two-Dimensional Fluorograms



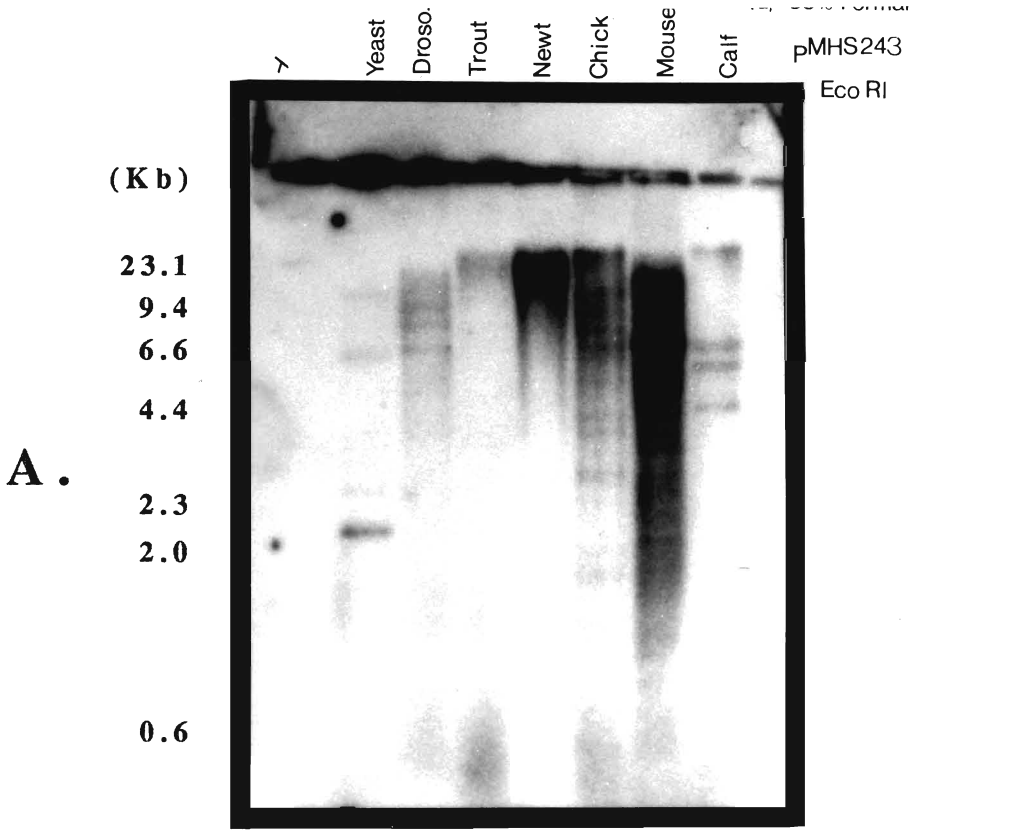
to the transcripts from the unamputated and heat shocked newts. Prior to Northern blot studies examining HSP70 mRNA accumulation, hybridization stringency conditions had to be determined for the heterologous murine hsp68 cDNA clone (pMHS-243). This probe shares 75% homology with most HSP70-like genes and can thus be used for cross hybridization reactions between species (Lowe and Moran, 1986). Furthermore, it not only detects the heat induced hsp70 mRNA but also cross-reacts with the constitutively expressed hsc70 transcripts in mammals (Lowe and Moran, 1986).

The optimal hybridization stringency was experimentally determined by testing the ability of the heterologous probe to cross-hybridize to genomic DNA from a variety of species on a Southern blot (Figure XIIA). Background binding of ^{32}P-labelled DNA probe to the nylon filter was eliminated when the wash temperature was raised to 55^{0}C. With the exception of trout, newt and lamda phage DNAs, Figure XIIA demonstrates the varying multiplicity of the HSP70-like gene copy numbers among diverse eukaryotes. The mouse hsp68 probe bound strongly to the homologous DNA from the mouse genome. To date, no data have been published indicating that lamda phage DNA contains endogenous HSP70 gene(s), thus this lane served as a negative control for hybridization. Lack of hybridization to the trout and newt may be due to poor DNA digestion by the restriction enzyme EcoRI (see Appendix for a figure of the ethidium bromide stained gel).

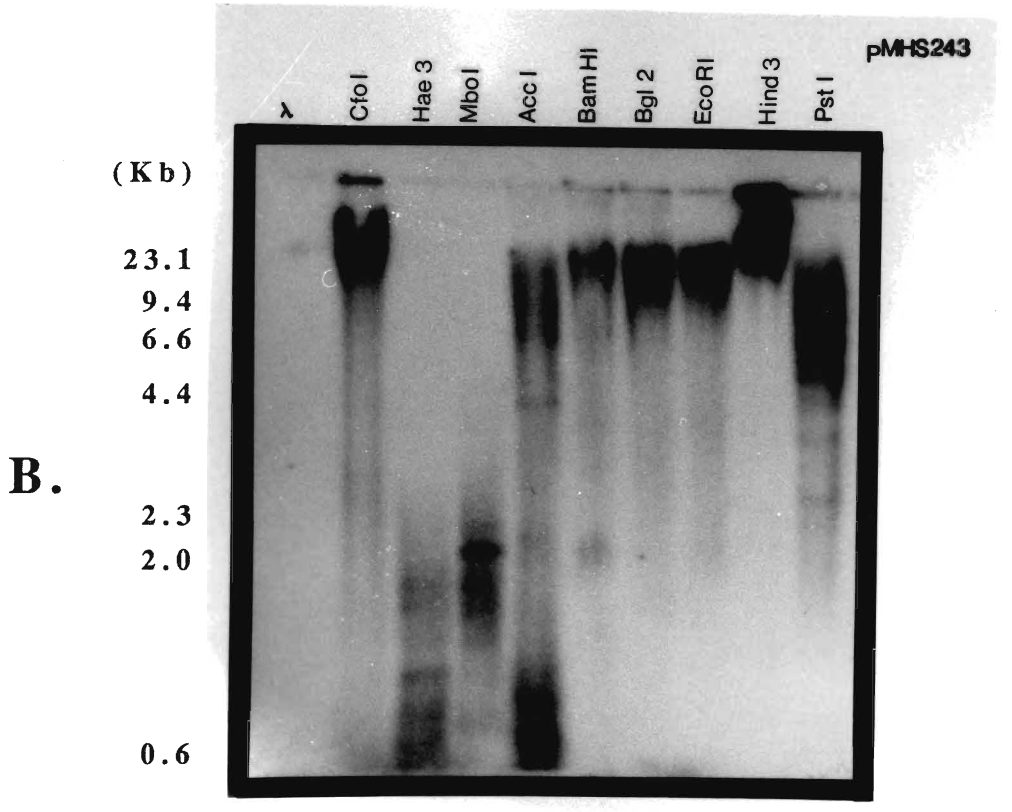
Figure XIIB illustrates the effectiveness of low stringency hybridization in visualizing HSP70 banding of a newt Southern blot probed with pMHS-243. To investigate this possibility, genomic DNA from the newt was digested with a variety of restriction enzymes to attempt to achieve complete digestion (see Appendix for a figure of the ethidium bromide stained gel) and a Southern blot prepared. The observed hybridization bands

Figure XII. Southern hybridization of DNA showing the presence of the HSP70-like genes in the genome of eukaryotic organisms. The blot was probed with the murine hsp68 cDNA gene (pMHS-243) at low stringency and exposed for 4 days at -70^{0}C. (A) represents DNA isolated from various organisms and digested with the restriction enzyme Eco R1. (B) represents DNA extracted from newt and digested with various restriction enzymes. Lambda Hind3 digested DNA molecular weight markers (Kb) are indicated.

Figure XII. HSP70 Probed Southern Autoradiograms



A.



B.

do not correspond to any repetitive sequences (shown in the ethidium bromide stained gel) and probably represent true hybridization due to their affinity for the probe at near maximum washing temperatures (5^0C below high stringency conditions). It appears that the HSP70 gene belongs to a multigene family in the newt. The actual copy number cannot be ascertained from this Southern blot nor can one distinguish the inducible, cognate or pseudogene composition. It was perhaps fortuitous that HSP70 gene hybridization was observed at all, since the *Notophthalmus viridescens* genome ($4.35x10^{10}$bp/haploid genome) contains 14.5 times more DNA than does the average mammalian genome ($3x10^9$bp/haploid genome), (Straus, 1971). Thus, while 10µg of genomic DNA is needed to detect a single copy gene in mammals under these hybridization condition; one would presumably need to load 145µg of DNA per lane to locate a single copy gene in *Notophthalmus viridescens* (Straus, 1971; Maniatis, 1982).

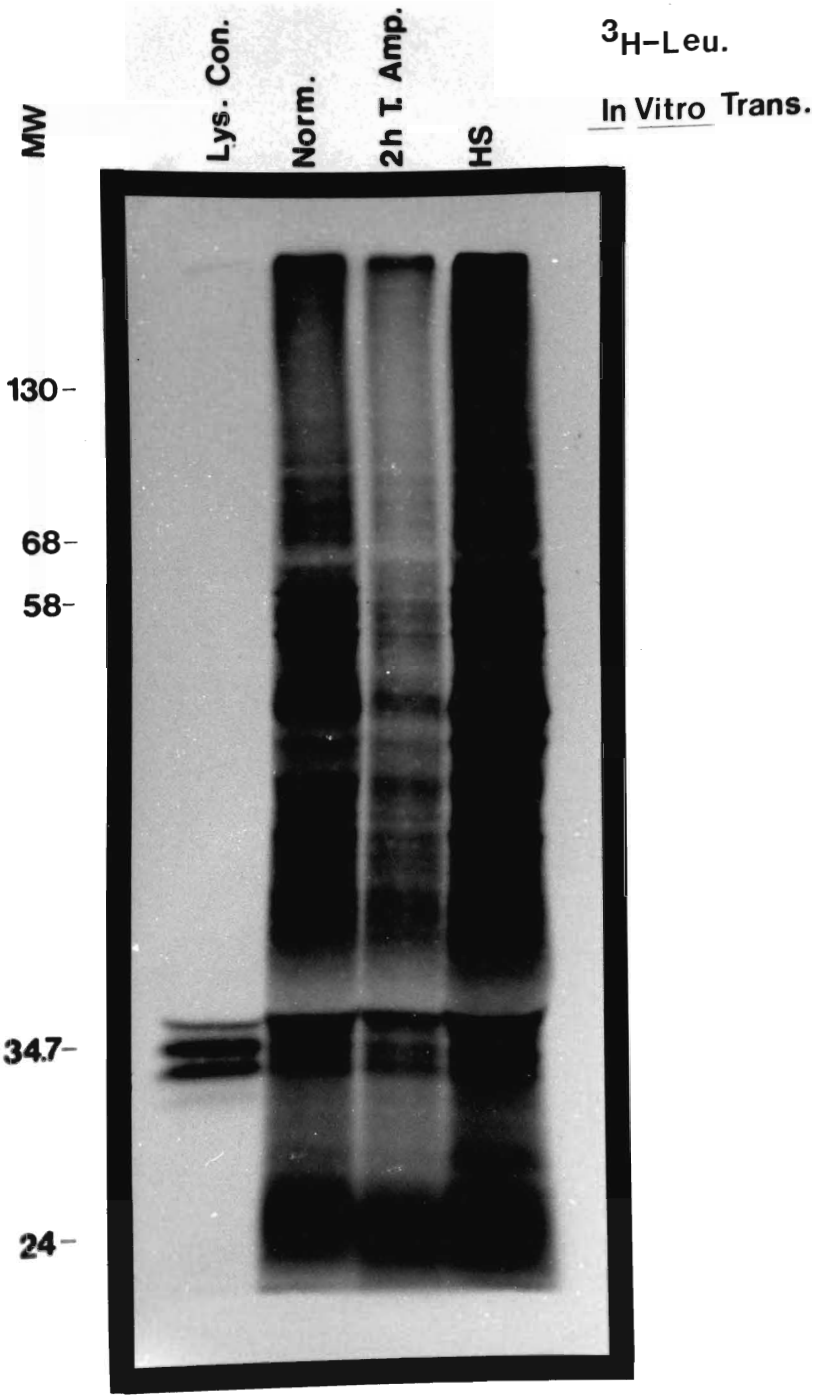
Poly(A)$^+$ RNA isolated from unamputated control, heat shocked and forelimb/hindlimb amputated newts was tested for its translatability prior to Northern hybridization. Evidence of its translatability was apparent in the form of *in vitro* translation products produced from poly(A)$^+$ mRNA relative to the lysate control (Figure XIII). Precise qualitative or quantitative analyses of peptide synthesis is difficult due to insufficient resolution and the apparent variance of cpm loaded per lane. Still, comparison of polypeptides synthesized from the rabbit reticulocyte cell-free system indicate only marginal differences between the control (19^0C) and heat shocked mRNA (34^0C). The lack of an abundant hsp70 protein band in the heat shock lane may be due to: 1) the preferential synthesis of lower molecular weight proteins intrinsic to the *in vitro* system (Colbert *et.al*, 1987) and/or 2) failure to use RNA prepared from polysomes. Induction of

Figure XIII. Isolated newt RNA was tested for its translatability in a rabbit reticulocyte lysate system. Evidence of its translatability was apparent in the form of *in vitro* tritiated translation products produced from poly(A)$^+$ mRNAs.

Lys. Con.= lysate control of rabbit reticulocyte
Norm.= unamputated control
2 h T. Amp.= 2 h post-amputation of total appendages
HS= heat shock (34.5^{0}C)

30,000cpm was loaded per lane and the flurogram was exposed for 7 days at -70^{0}C.

Figure XIII. In Vitro Translation Products

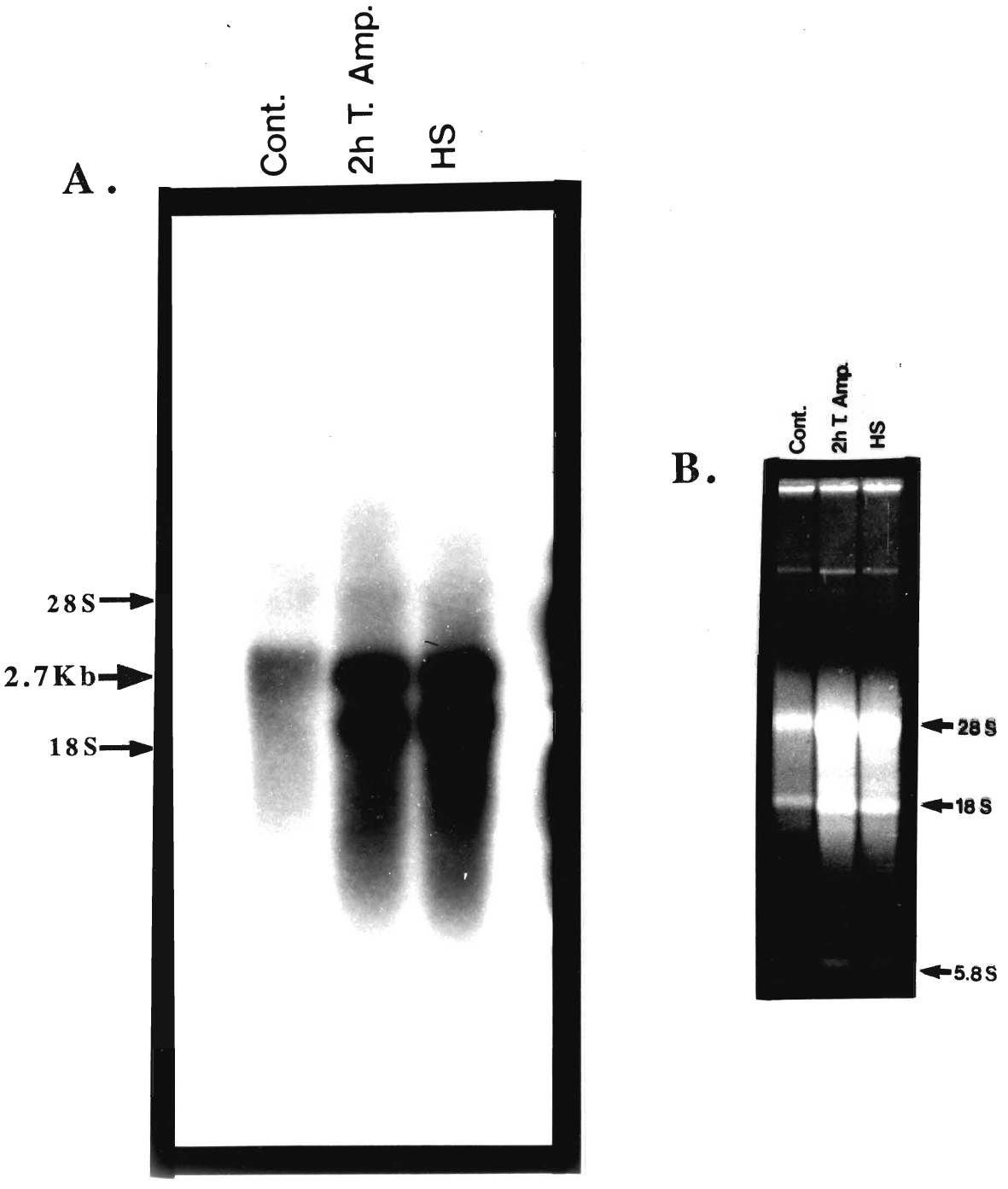


hsp synthesis cause the reassociation of polysomes that have a high affinity for hsp mRNAs. Hence dilution of hsp mRNAs incurred by total RNA extraction would be minimized by polysomal RNA isolation (Bienz and Gurdon, 1982) and/or 3) the extreme instability and autohydrolysis of hsp mRNA and hsp70 protein, respectively (Mitchell *et.al*, 1985). Protein products synthesized from the total poly(A)$^+$ mRNA extracted from the whole newt at 2 hours after amputation of all appendages revealed no prominent hsp banding patterns (Figure XIII). Isolation of poly(A)$^+$ mRNA from distal stump and tail regenerates would diminish the possible dilution of any prevalent regeneration-specific mRNAs present in total RNA extracted from whole animals. Regeneration-specific RNA preparations would facilitate, perhaps, the unambiguous detection of preferential transcripts and thus their protein products.

Size characterization and enhanced transcription of the HSP70 gene(s), as determined by Northern hybridization is demonstrated in Figure XIVA. Both heat shock and simultaneous amputation of all appendages caused increased expression of HSP70 transcripts in the whole animal relative to the control. The approximate sizes for the newt HSP70 transcripts are 2.7Kb and 2.6Kb respectively. One could speculate that the smaller transcript belongs to the constitutive hsc70 class since in other species, the inducible hsp70 transcripts are larger than hsc70 mRNAs due to their long 3' untranslated regions (Lowe and Moran, 1986). The absence of this lower band and the presence of the larger 2.7Kb mRNA in the control lane argue against this possibility however. Neither the newt HSP70 gene family nor its transcripts have been characterized in detail and therefore, one cannot rule out the possibility that the smaller transcript may actually be from another inducible heat shock gene while the larger transcript is composed of both

Figure XIV. (A) represents Northern hybridization showing the effects of a one hour heat shock and total appendage amputation on the accumulation of HSP70 transcripts. The blot was probed with the murine hsp68 cDNA gene (pMHS-243) at low stringency and exposed for 4 days at -70^{0}C. (B) represents the RNA (20µg) agarose gel (0.8%) stained with ethidium bromide and photographed under UV light.

Figure XIV. Northern Autoradiogram and Ethidium Gel



cognate and inducible transcripts of equivalent sizes. These data do however, indicate that the trauma of mechanical injury induces HSP70 transcription in a manner analogous to that stimulated by hyperthermia.

Hybridization with an actin probe (e.g. chick actin; pA1) was not used to verify that equivalent amounts of poly(A)$^+$ mRNA were applied to the Northern blot or to test for RNA integrity. Heikkila *et.al.* (1985, 1987) have observed differential expression of actin genes in developing species (e.g. *Xenopus* oocytes) after heat shock. Confirmation of quantities of RNA applied to the gel was judged by ethidium bromide staining (Figure XIVB). The RNA appeared not to be degraded since 1) the RNA was translatable *in vitro* (Figure XIII) and 2) the 28S and 18S rRNA transcripts were intact and present in appropriate ratios (2:1) in each preparation (Figure XIVB).

No quantization of relative mRNA levels was done by scintillation counting of the radiolabelled bands. Furthermore, S1 nuclease protection assays to verify that the transcripts arose from the same hsp68 gene that was used as the radiolabelled probe were not performed.

Regeneration Stage-Specific Accumulation of HSP70 as Determined by Immunoblotting

Western blot analyses were performed using a murine monoclonal antibody (N27) prepared against the human cognate and inducible HSP70 forms (i.e. hsc73 and hsp72, respectively), (Welch and Feramisco, 1984). Figure XVA appears to reveal a slight increase in the level of HSP70 in limb tissue of heat shocked newts when compared to the basal accumulation in the unamputated control limb. This slight HSP70 increase is in contrast to the 200% increase observed in the 70kDa protein (hsp70) seen in the fluorograms of Figures IXA and XIB. From these results it appears that monoclonal antibody N27 fails to cross-react to a significant extent with the

highly heat inducible form of hsp70 of the newt. In support of this contention, amputation resulted in a measurable loss of hsp70, presumably the constitutive form (hsc70), from stump tissues during the first 24 hours after amputation (Figure XVA). In contrast to the decrease in HSP70 in the distal limb stump, no loss of HSP70 was observed in the corresponding tail tissues.

By the early bud stage, HSP70 in the blastema appears to be higher than in unamputated/unstressed limbs (Figure XVB) and considerably more abundant than during the early wound healing stages of the distal limb stump (Figure XVA). Levels decline slightly after the late bud stage, ultimately reaching control values by the early to late digit stage. Low levels of non-specific cross-reactivity of N27 to newt antigens of molecular weights between 40kDa and 50kDa was shown to be present in both immunoblots (Figure XVA/B).

To determine if the secondary conjugated antibody (GAM-AP) cross reacted with the HSP70 antigen, a Western blot was performed in the absence of primary antibody (N27). Examination of Figure XVI reveals no significant binding under these conditions. Thus the results shown in Figure XV are most likely due to the specific binding of the N27 monoclonal antibody to HSP70.

In a second series of experiments (see Appendix), no binding to hsp70 was obtained when immunoblots were probed with another murine monoclonal antibody (C92), specific to the human inducible hsp72 protein (Welch and Feramisco, 1984). In addition, this antibody showed an unacceptable degree of non-specificity in its binding with a number of other newt antigens. This non-specific cross-reactivity was not attributable to non-specific binding of the secondary conjugated antibody (GAM-AP). The lack of

Figure XV. Western immunoblot of 10% SDS-PAGE probed with the monoclonal antibody (N27) which recognizes both the inducible and constitutive forms of human HSP70. (A) represents the effects of a one hour heat shock and amputation through the humerus on HSP70 accumulation. (B) represents the stage-specific levels of HSP70 in the regenerating forelimb of the newt quantitated by Western immunoblot as described in (A) above. The molecular weight markers are expressed in kDa.

(A) Cont.= unamputated control forelimb
HS= heat shock (34.5^{0}C)
1 h T= 1 h tail (p.f.a.)
1 h L= 1 h distal limb stump (p.f.a.)
6 h T= 6 h tail (p.f.a.)
6 h L= 6 h distal limb stump (p.f.a.)
1 d T= 1 day tail (p.f.a.)
1 d L= 1 day distal limb stump (p.f.a.)

(B) Cont.= unamputated control forelimb
HS= heat shock (34.5^{0}C)
EB= early bud
LB= late bud
ED= early digits

Note, 20µg of protein was loaded per lane.

Figure XV. Stage-Specific Western Hybridization

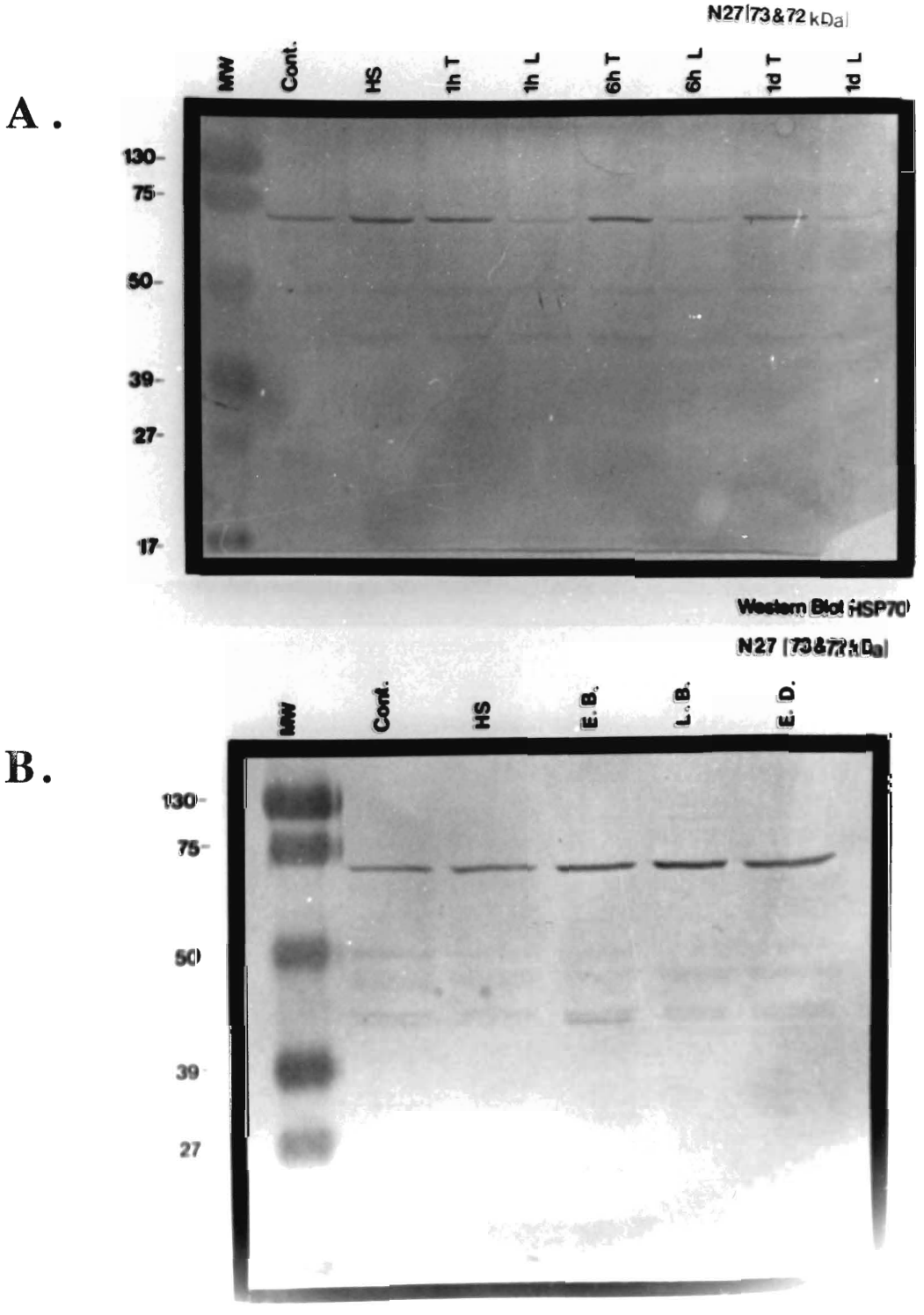
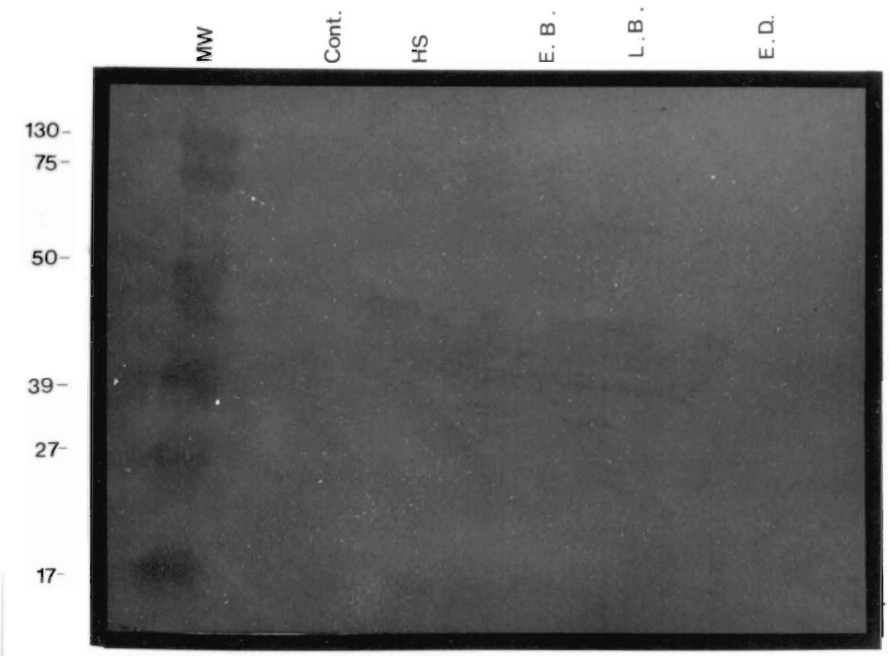


Figure XVI. Western immunoblot of 10% SDS-PAGE to ascertain the specificity of the secondary conjugated antigen (GAM-AP) for the HSP70 antigen in the absence of the primary antibody (N27). Note the lack of hybridization in the heat shock and regenerate stages.

Cont.= unamputated control forelimb
HS= heat shock (34.5^{0}C)
EB= early bud
LB= late bud
ED= early digits

20µg of protein was loaded per lane.

Figure XVI. Western Hybridization Control Blot



binding between C92 and the heat inducible newt hsp70 antigen was not apparently due to poor electrophoretic transfer of 70kDa proteins, as evidenced by the degree of Amido black staining of the nitrocellulose. The antigenic determinant (epitope) on the heat inducible hsp70 may not be recognized by the monoclonal antibodies (N27 and C92).

Changes in Ubiquitin Levels Are Not Associated With Regeneration Stage-Specific Accumulation of HSP70 as Determined by Immunoblotting

Hyperthermia has been shown to independently activate ubiquitin expression due to the HSE sequence located within the 5' promoter region of the ubiquitin genes (Bond and Schlesinger, 1986). Synthesis of ubiquitin in mammalian cells, within the first 2 h of temperature elevation, plays a major role in the selective degradation of aberrant proteins induced by hyperthermia (Parag *et.al*, 1987). Furthermore, it has been proposed that the ubiquitin system is coupled to induced HSP gene activation (see Literature Review).

To examine if the stage-dependent accumulation of HSP70 and the early wound induced expression of amp70 was associated with changes in the levels of ubiquitin, immunoblot analyses were conducted using a murine monoclonal antibody (Ub1) directed against the human ubiquitin protein (see Appendix). A low level of ubiquitin was detected in samples prepared from the limbs of both heat shocked and unstressed control newts. A slight increase in ubiquitin accumulation was detected at the late bud stage; the same stage in which a slight elevation of HSP70 accumulation was evident (Figure XV). No difference in ubiquitin levels were apparent, relative to the control, in preparations from the tail or limb during the early wound healing period. From these data, it is difficult to draw a link between ubiquitin levels and HSP70 levels in the tissues of the regenerating limb. Furthermore, the

monoclonal antibody used in this study may cross-react with both the conjugated and unconjugated forms of ubiquitin (Showalter, personal communication). The recent acquisition of a ubiquitin cDNA probe may more readily allow for a detailed analysis of the possible coordinate synthesis of ubiquitin, amp70 and HSP70 in the regenerating amphibian limb.

Discussion

Although it has been reported that there are stage-dependent qualitative and quantitative differences in the soluble protein content of adult newt forelimb regenerates (Dearlove and Stocum, 1974; Garling and Tassava, 1984), little information is available on the initial protein composition prior to wound epithelialization. In the present study, the effects of hyperthermia and mechanical injury (limb amputation) on the early synthesis and accumulation of soluble proteins in distal stump tissues of regenerating limbs of *Notophthalmus viridescens* were examined.

In initial experiments, striking similarities were detected between the array of soluble proteins in the distal stump tissues of amputated newt forelimbs with those found after heat shock. In both cases, there was a general and reversible repression of the synthesis of most forelimb proteins and a transient increase in specific protein synthesis. The most significant increase in a specific protein species synthesized under each stress was that of a 70kDa protein(s). This protein(s), initially presumed to be HSP70 in both cases, was rapidly induced systemically after heat shock of the whole newt, but was synthesized locally in the distal stump tissue of the limb in response to amputation. Previous studies (at 3 days post-amputation and later) revealed no amputation induced accumulation of a 70kDa protein in either denervated or innervated forelimb regenerates (Dearlove and Stocum, 1974; Slack, 1982; Garling and Tassava, 1984; Bao *et.al*, 1986).

Heikkila and Schultz (1984) demonstrated that mechanical injury of rabbit blastocysts caused increased synthesis of a 70kDa protein that they suggest is identical to heat inducible hsp70. In a related study, Currie and White (1981) have reported that the act of slicing various mammalian tissue, induces the preferential synthesis of a 71kDa protein (P_{71}) having an acidic

isoelectric point. Fujio *et.al.* (1987), by peptide mapping, identified the protein as hsp70.

Concomitant with the increased synthesis of a 70kDa protein in the distal limb stump at one hour post-amputation, we detected an increased synthesis of a 30kDa protein. The accumulation of this 30kDa protein parallels the increase in hsp30 synthesis observed in the forelimbs of heat shocked newts. Hsp30, like hsp70, has been shown to have a developmentally regulated, stage-dependent expression that may have an independent mechanism of regulation from that of hsp70 (Heikkila *et.al*, 1986; Heikkila *et.al*, 1987; Krone and Heikkila, 1988). Although no kinetic analysis was undertaken in this study, the temporal pattern of the coordinate 70kDa and 30kDa syntheses and repression parallels that of other reports (Kothary and Candido, 1982; Bienz, 1984a; Easton *et.al*, 1987; Rutledge *et.al*, 1987; Krone and Heikkila, 1988).

Two-dimensional gel analysis of the major forelimb 70kDa protein(s) of the heat shocked and amputated newts revealed biochemical differences in these proteins induced by the different stresses. In control limbs (unamputated, 19^0C) a relatively low level of synthesis of the presumed constitutive hsc70 protein (i.e. hsc73 in humans) was observed. Similar analysis of the corresponding limb proteins one hour after heat shock (34.5^0C) showed a marginal increase in hsc70 accumulation and relatively high levels of synthesis for three heat inducible hsp70 proteins (i.e. hsp72-like proteins in humans). Hsp70 resolved into three isoelectric variants with pIs in the range of 6.5 to 6.8. The heterogeneity of the hsp70 stress proteins has been documented in a variety of species, though never in the urodele, *Notophthalmus viridescens* (Burdon, 1986; Lindquist and Craig, 1988). Welch and Feramisco (1984) and Welch and Suhan (1986) have found that

the number and relative amounts of the hsp70 isoforms vary somewhat between different cell types and are dependent on the type of inducer used to elicit the stress response.

In contrast to the heat shock induced synthesis of the hsp70s, very little synthesis of hsc70 or hsp70 proteins was observed in the distal limb stump at one hour post-amputation. The prominent band of 70kDa protein(s) observed in the one-dimensional SDS-PAGE fluorograms migrated in the two-dimensional fluorogram as a single 70kDa protein (amp70) of acidic pI that was absent in limb tissue of the heat shocked newt. The relationship of amp70 to that of the HSP70-like family remains to be determined. Peptide mapping, immunological studies and ATP-binding assays may shed light on this possible relationship. Perhaps amp70 is an acidic member of the multigene HSP70 family with a separate mechanism of regulation controlled by physical trauma. Alternatively, the stress of mechanical injury may induce a covalent modification of an HSP70 protein(s) creating an isoform with an acidic pI. Post-translational modifications such as phosphorylation and methylation have been described for the HSP70s and could possibly account for the heterogeneity observed among species (Burdon, 1986; Lindquist and Craig, 1988). A final possibility is that this protein may prove to be unique and unrelated to the HSP70-like family.

To address the relationship of amp70 to HSP70-like proteins, Northern blot hybridization was conducted with the mouse hsp68 cDNA probe, pMHS-243, which cross-reacts to both the inducible and constitutive HSP70 murine genes (Lowe and Moran, 1986). Increased levels of HSP70 mRNAs were expressed in whole animals in response to heat shock and in forelimb and hindlimb tissues one hour after amputation when compared to the unamputated control limb levels. Two HSP70 mRNAs, of 2.7Kb and 2.6Kb

respectively, were identified. The size of these HSP70 transcripts are similar to values reported for a number of species (Heikkila *et.al*, 1986; Heikkila *et.al*, 1987; Krone and Heikkila, 1988). Although the results are not be strictly quantitative due to slight variations in the amount of RNA loaded per lane (see Figure XIVB), they probably reflect hsp70 induction due to the detection of the 2.6Kb transcript which is absent in the unamputated control lane. Thus the ability of the newt to synthesize the proteins hsp70(s) and amp70 was correlated with increased HSP70 mRNA synthesis. The similar mRNA synthetic patterns induced by amputation and heat shock, indicate that amp70 is perhaps a member of the highly conserved HSP70 multigene family. This result does not preclude the possibility that the HSP70 mRNA is post-transcriptionally regulated and not translated as is observed in *Xenopus* oocytes (Bienz and Gurdon, 1982; Baltus and Hanocq-Quertier, 1984). Until specific HSP70 genes are isolated for the newt, the problem of distinguishing between the multiple species of HSP70 mRNA will remain.

With the increased resolution of two-dimensional gel electrophoresis, it was revealed that basal levels of amp70 were present in the unamputated control forelimb and in the tail after limb amputation. This observation raises the possibility that the accumulation of amp70 in the limb after amputation may not be due to *de novo* synthesis in the stump induced by this trauma, but may result from transport to the site as a response to wound healing. In support of this concept, it has been demonstrated that human lymphocytes express hsp70 after mitogenic stimulation (Colbert and Young, 1987; Kaczmarek *et.al*, 1987; Ferris *et.al*, 1988; Haire *et.al*, 1988).

Although preliminary immunohistofluorescent studies (results not shown) using a mouse monoclonal antibody (N27) directed against the human hsp72/hsc73 antigens failed to resolve this dilemma, arguments in

favour of *de novo* 70kDa synthesis in the limb stump can be made. First, histological evidence demonstrates that erythrocytes, phagocytic leukocytes (chiefly granulocytes) and melanocytes do not appear in the site until the onset of epithelialization, two to four hours after amputation in the newt (Repesh and Oberpriller, 1978, 1980; Schmidt, 1968). In addition, neutrophils, the predominant cells associated with the wound, do not appear in the site until the first few days of the inflammatory response (Sicard, 1985). Other leukocytes (e.g. phagocytic macrophages and lymphocytes) are only apparent as the inflammatory response progresses to day five post-amputation (Sicard, 1985). Secondly, if the accumulation of amp70 at the wound site is due to its production of circulating leukocytes, one would expect a transient accumulation of this 70kDa protein in tail stumps after the amputation of the tail analogous to that of the distal limb stump after its amputation. Preliminary experiments by H. Karn (in our laboratory) failed to show increased synthesis of a 70kDa protein in distal tail stump sections within the first 3 hours post-amputation. This apparent difference in the response of tail and limb tissue to wounding is an intriguing observation which may reflect alternative mechanisms by which these tissues undergo epimorphic regeneration.

Amp70 protein accumulation in the limb stump happens within the first hour after forelimb amputation, a time period when haemostasis occurs. The rapid mechanism of blood clotting in the urodeles is still poorly understood (Schmidt, 1968; Wallace, 1981; Sicard, 1985). It is possible that amp70 may be a coagulator factor involved in haemostasis or in the formation of the moist fibrin network which forms at the wound surface at this time (Repesh and Oberpriller, 1978, 1980). In mammals, during cessation of blood flow subsequent to wounding, only prothrombin has a

molecular weight approximating that of amp70 (Tizzard, 1988). However, since amp70 does not accumulate in tail stumps, it is unlikely that prothrombin or any other enzymes of the clotting cascade represent the source of the 70kDa protein in the limb stump. Thus amp70 appears to be synthesized *de novo* in the forelimb stump in response to amputation and is not transported to the wound site as a mechanism of wound healing.

Results from immunoblotting studies on the developmental regulation of HSP70 synthesis are confounded by the apparent lack of cross-reactivity of antibody N27 with the heat inducible hsp70(s) of the newt. Although it cross-reacts with both forms of human HSP70 (Dr. W. Welch, personal communication) and strongly reacts with a 70kDa antigen in unstressed cells of the newt, heat shock only marginally increased the titre of the protein recognized by this particular antibody. This marginal increase contrasts the high heat inducible levels of the hsp70 isoforms evident in Figure XIB but is in agreement with studies where a slight accumulation (under two-fold) for the constitutive hsc70 protein upon temperature elevation was observed (Palter *et.al*, 1986; Pelham, 1986; Dworniczak and Mirault, 1987). Thus the results obtained by immunoblotting with N27 most likely represented the regulation of the constitutive newt hsc70 antigen and not the inducible hsp70(s) form.

Regulation of the levels of constitutive hsc70 and other HSPs occurs not only in response to temperature elevation but during normal embryogenesis of organisms implicating a role for these HSPs in development (Morange *et.al*, 1984; Heikkila *et.al*, 1985; Heikkila *et.al*, 1986; Palter *et.al*, 1986; Bonato *et.al*, 1987; Lindquist and Craig, 1988). Although the mechanism by which HSP70-like proteins function is still unclear, it has

been strongly suggested that they proteins stabilize aberrant or nascent proteins (Pelham, 1986).

Immuno-blotting results with N27 (see Figure XV) support a developmental or regeneration stage-dependent expression of hsc70 synthesis in the regenerating limb of the newt. A slight increase of hsc70 at the cellular 'dedifferentiation' stage (early bud) compared to unamputated limbs agrees with data obtained from undifferentiated embryonal carcinoma (EC) cell lines (Morange *et.al*, 1984; Wittig *et.al*, 1983; Mezger *et.al*, 1987) and the pluripotent pre-implantation mouse embryos (Lowe and Moran, 1984; Curi *et.al*, 1987).

The initial slight increase of hsc70 in the early bud regenerate is probably due to the asynchronous and asymetric proliferation of blastema cells. At this stage only 26% of the 'dedifferentiated' blastema cells are actively progressing through the cell cycle (Goldhamer and Tassava, 1987; Tassava *et.al*, 1987; Tomlinson and Barger, 1987).

The levels of hsc70 peak at the mid-late bud stage and then decline to the unamputated control levels by the late digit stage. This peak of blastema hsc70 during the late bud stage, a period of intense mitotic activity, agrees with results by Ferris *et.al.* (1988) and Haire *et.al.* (1988) relating mitogen induced increases in constitutive hsc70 (four-fold) with increases in mitotic activity of human lymphocytes. The decline of hsc70 observed after the late bud stage may reflect a shift in the state of differentiation of blastema cells reminiscent of that induced by retinoic acid (RA) in F9 teratocarcinoma cells of the mouse (Imperiale *et.al*, 1984). At this period a proximodistal wave of redifferentiation occurs in the proliferating blastema cells (Wallace, 1981). Wier and Scott (1986) reported differential protein synthesis in the RA induced transition of non-terminal 3T3 mesenchymal stem cells to

terminally differentiated cells. Although the modulated proteins have not been characterized during this transition, it would be of great interest to conduct a Western blot with N27 to see if hsc70 was being influenced similar to the late digit stage of the newt forelimb regenerate.

In contrast to the later stages of blastema development (i.e. EB, LB and ED), hsc70 levels declined dramatically at 1 h post-amputation and were maintained at lower than control levels for a period of at least 24 h. This decrease in hsc70 after amputation was localized to the distal limb stump and was not correlated with a systemic decrease in hsc70. Interestingly, amp70 was shown to be inversely regulated with hsc70 in the distal limb stump of post-amputated newts. Two-dimensional fluorograms (Figure XID) substantiate the loss of hsc70 synthesis in the distal limb stump after forelimb amputation.

Although no precedents in the literature can be found for the repression of hsc70 synthesis due to mechanical injury, preliminary data in our laboratory indicate that intraperitoneal injection of retinoic acid (RA) has a similar effect on hsc70 levels in the blastema. Retinoic acid results in a dose-dependent proximodistal reduplication when introduced into regenerating limbs of amphibians (Maden, 1983; Crawford and Stocum, 1988). Analogous to amputation, RA induces the transient and localized accumulation of a 70kDa protein (of unknown pI) that does not have a common antigenic determinant recognizable by the N27 human monoclonal antibody. Indeed, as determined by immunoblotting with N27, RA appears to cause a decrease in the levels of hsc70 in these tissues, similar to the response of limb tissues to amputation. An interesting possibility is that RA causes a "reprogramming" of "positional information" in blastema cells which render them in a state equivalent to that found within the first 24 hours

after amputation. When the effects of RA wear off, however, the positional information, in the blastema, needs to be somehow readjusted to give proximodistal duplication instead of just intercalation. That retinoic acid can induce differential expression of HSPs with the concomitant repression of several non-stress proteins in the fetal mouse limb bud indirectly supports these early results in the newt regenerate (Anson *et.al*, 1987). Though the full scope of genes affected by RA is unclear (Wang *et.al*, 1985; Horton *et.al*, 1987; LaRosa and Gudas, 1988), it is known that RA mediates alterations in genome expression through the formation of a cellular retinoic acid binding protein-RA receptor complex which behaves much like a steroid hormone-receptor complex (Petkovich *et.al*, 1987; McCormick *et.al*, 1988).

In summary, both heat shock and forelimb amputation caused a decrease in total protein synthesis concurrent with a preferential increase in the synthesis of 30kDa and 70kDa proteins. Two-dimensional gel electrophoresis revealed that the amputation induced 70kDa protein of the distal limb stump (amp70) was unique and distinct from the more basic heat inducible hsp70 isoforms. Northern blot analysis using a murine hsp68 cDNA probe showed that mRNA transcripts, induced by forelimb/hindlimb amputation, were analogous to those induced by heat shock. Finally, during epimorphic regeneration of the forelimb, there was a stage-dependent accumulation of the putative hsc70. The titre of hsc70 rises gradually after the wound healing stage and eventually increased to a level greater than controls during the late bud stage of regeneration.

In order to determine the role of amp70 induced by the mechanical stress of amputation and the function of HSP70 (hsc70/hsp70) in the regenerating newt blastema, future molecular experiments will be required. These might include:

1) Characterization of the biochemical relationships between amp70, the RA induced 70kDa protein, and other members of the HSP70-like family by two-dimensional gel electrophoresis and peptide mapping. Analysis, of amp70, by affinity chromatography on ATP-agarose gels (Welch and Feramisco, 1984) could determine if amp70 contains an ATP binding domain similar to the HSP70-like family.

2) Isolation of amp70 monoclonal or polyclonal antibodies which could facilitate further characterization of the role of amp70 protein during early limb wound healing. These results may help to understand if amp70, in the blastema, is stage-dependent, tissue-specific and synthesized *de novo*. Screening of a newt λgt11 expression cDNA library with anti-amp70 antibody may allow for the isolation of the amp70 cDNA. *In situ* hybridization using radiolabelled amp70 probes could then be used to localize amp70 transcripts within the cells of the blastema after stress treatments.

3) Isolation and sequence analysis of newt-specific amp70 and HSP70 genes from a newt genomic (Dr. J. Brockes) or λgt11 expression library could provide information regarding their nucleotide homology and regulation. Correlation of HSTF and ubiquitin transcription with amp70 synthesis may determine if the mechanism of amp70 regulation is similar to that of HSP70. Furthermore, characterization of any transcriptional and/or translational controls of amp70 or HSP70 (e.g. hsc70) synthesis could be elucidated by using protein and transcriptional inhibitors.

4) Denervation, in the limb, of the brachial nerves would address whether amp70 and/or HSP70 synthesis in the blastema is nerve-dependent.

5) DNA binding domain properties of amp70 could be assayed by footprinting to assess its possible regulatory role in gene expression.

6) Comparison of Western blot and immunohistochemical studies using available HSP70 monoclonal or polyclonal antibodies should allow further characterization of HSP70 proteins after heat shock and during limb regeneration in the newt. These results may help to understand the function of the stage-dependent and tissue-specific intracellular localization of HSP70s (Welch and Suhan, 1986; Welch and Mizzen, 1988). Evidence by *in situ* hybridization using radiolabelled HSP70 probes may also be used to localize HSP70 transcripts within the cells of the blastema after stress treatments.

7) Further characterization of other stress induced proteins of lower molecular weight (e.g. hsp30) in the heat shocked and 1 h post-amputated newts would also be of interest.

Conclusions

The ubiquitous heat shock proteins play a protective role in maintaining cell viability during the deleterious effects of heat and other stresses as well as a developmental role during embryogenesis. In this thesis, heat shock proteins were studied in *Notophthalmus viridescens* in response to the mechanical injury of forelimb amputation and during its subsequent epimorphic regeneration. The results indicate that amputation mimics the heat shock response quantitatively and temporally in its transient repression of the synthesis of most normal cellular protein complement and qualitatively in the localized expression of unique proteins (hsps?). Although limb amputation resulted in an increase in the synthesis of HSP70 mRNA analogous to that induced by heat shock, the amputation induced 70kDa protein (amp70) was determined to be distinct from newt hsp/hsc70 isoforms. In addition, amp70 did not antigenically cross-react with murine monoclonal antibodies directed against both the inducible and cognate HSP70 proteins of the human. Finally, the levels of the constitutive form of HSP70 (hsc70) were found to be regulated in a stage-dependent manner in the distal stump tissues of the regenerating forelimb of the newt. The highest levels were found in the mid-late bud stage, a period during which rapidly dividing blastema cells begin to redifferentiate in a proximodistal direction, while immediately after amputation, hsc70 synthesis was depressed below steady-state levels measured in the unamputated limb.

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Appendix

Figure XVII. Schematic representation of the recombinant HSP70 Plasmids. (A) is the murine cDNA probe, pMHS-243, blunt end ligated into the vector pEMBL8. A 1.4Kb PstI cDNA insert, containing a 5' open reading frame (black box) and a 3' untranslated region (white box) was isolated for ^{32}P-dCTP radiolabelling. (B) is a *Xenopus* 2.2Kb genomic probe (black box) containing 5' promoter sequences. The insert was cloned into Xho I restriction site of the vector pUC12.

Figure XVII. Recombinant HSP70 Plasmids

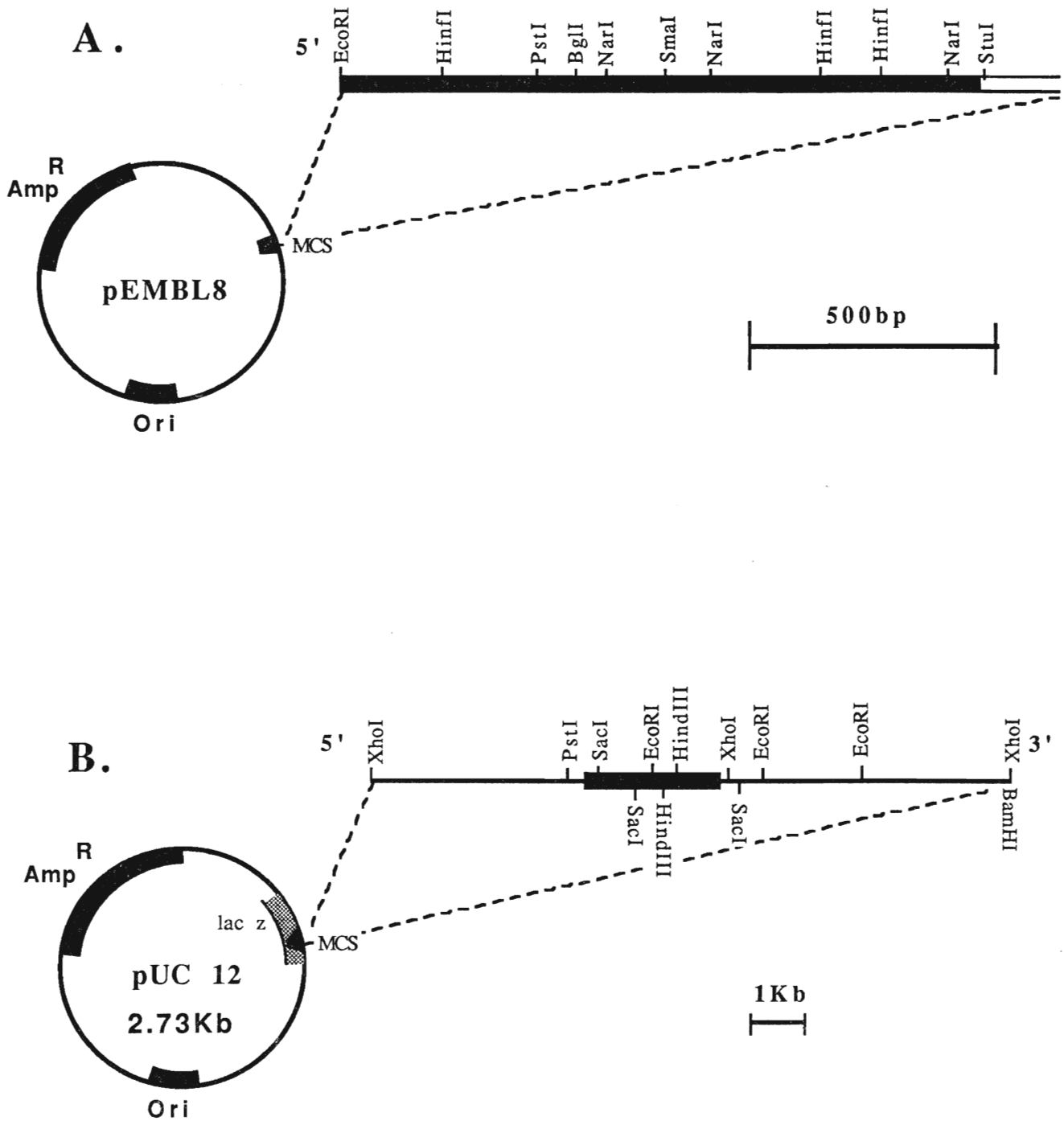


Figure XVIII. Graphical representation, corresponding to Figure IXB, of the absolute level of radiolabelled amino acid incorporation into the TCA-insoluble protein fraction. Protein peaks are expressed as a percentage of total integrated area.

(A) unamputated control forelimb
(B) heat shock
(C) 1 h tail (p.f.a.)
(D) 1 h distal limb stump (p.f.a.)

Proteins corresponding to 70kDa, 46kDa and 30kDa are indicated by arrows.

Figure XVIII. Relative Peak Abundance per Protein

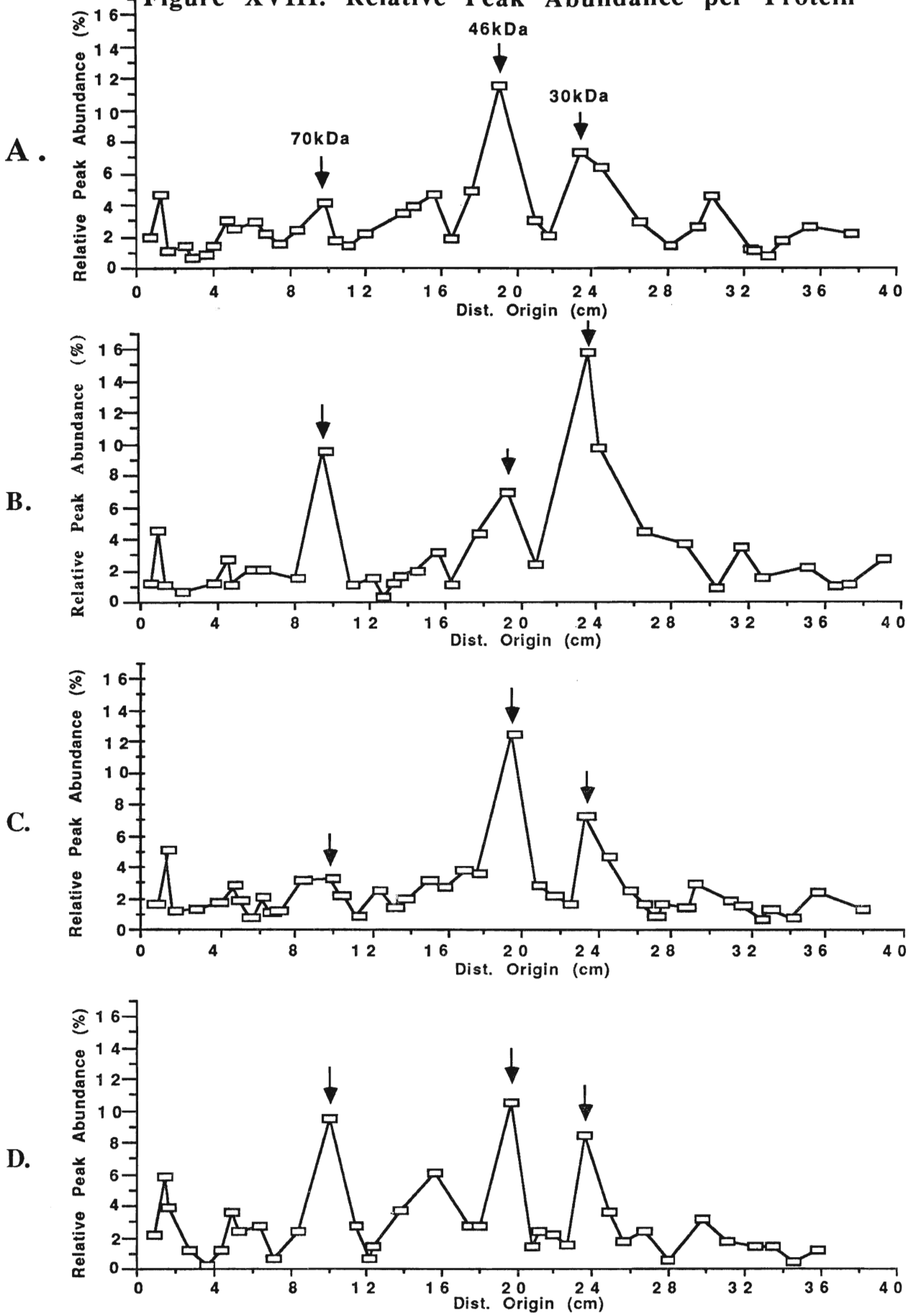


Figure IXX. Coomassie blue stained isoelectric focussing tube gels. 5,000cpm were loaded per IEF (3.5-10/6-8 ampholytes) gel and separated prior to second dimensional 10% SDS-PAGE. IEF standards (pI) corresponding in descending pH are equine myoglobin (3 bands), human carbonic anhydrase, bovine carbonic anhydrase, ß-lactoglobulin B and phycocyanin.

(A) unamputated control limb (13.7µg)
(B) 34.5^{0}C heat shock (35.92µg)
(C) 1 h tail (p.f.a.), (21.81µg)
(D) 1 h distal limb stump (p.f.a.), (41.71µg)

Figure IXX. Coomassie Stained Isoelectric Focusing Tube Gels

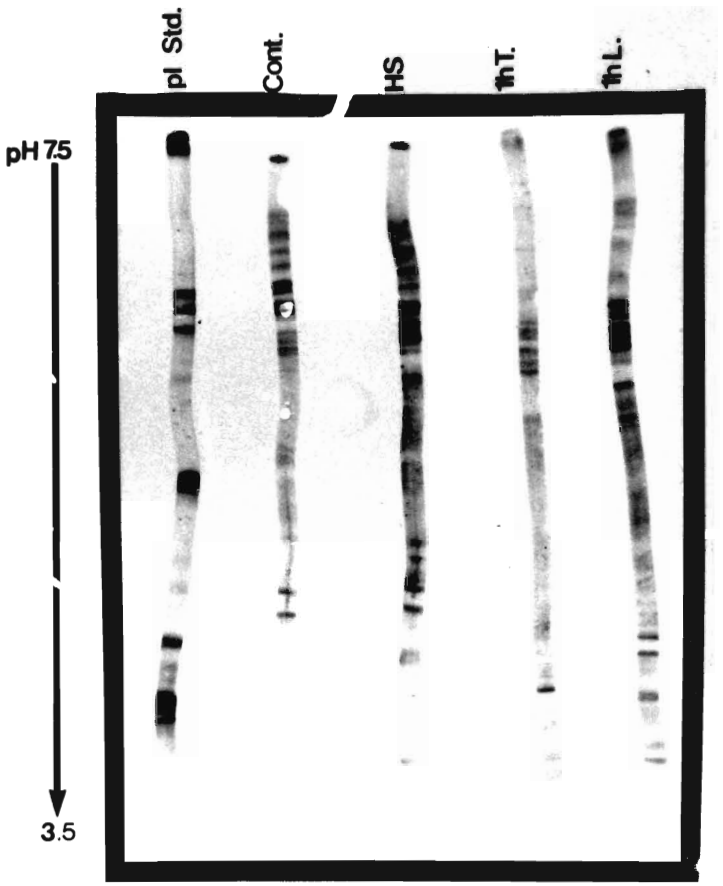


Figure XX. Silver stained two-dimensional 10% polyacrylamide gels, corresponding to the fluorograms of Figure XI, showing the effects of one hour heat shock and bilateral forelimb amputation.

(A) unamputated control limb (13.7µg)
(B) 34.5^{0}C heat shock (35.92µg)
(C) 1 h tail (p.f.a.), (21.81µg)
(D) 1 h distal limb stump (p.f.a.), (41.71µg)

Figure XX. Two-Dimensional Silver Stained Gels

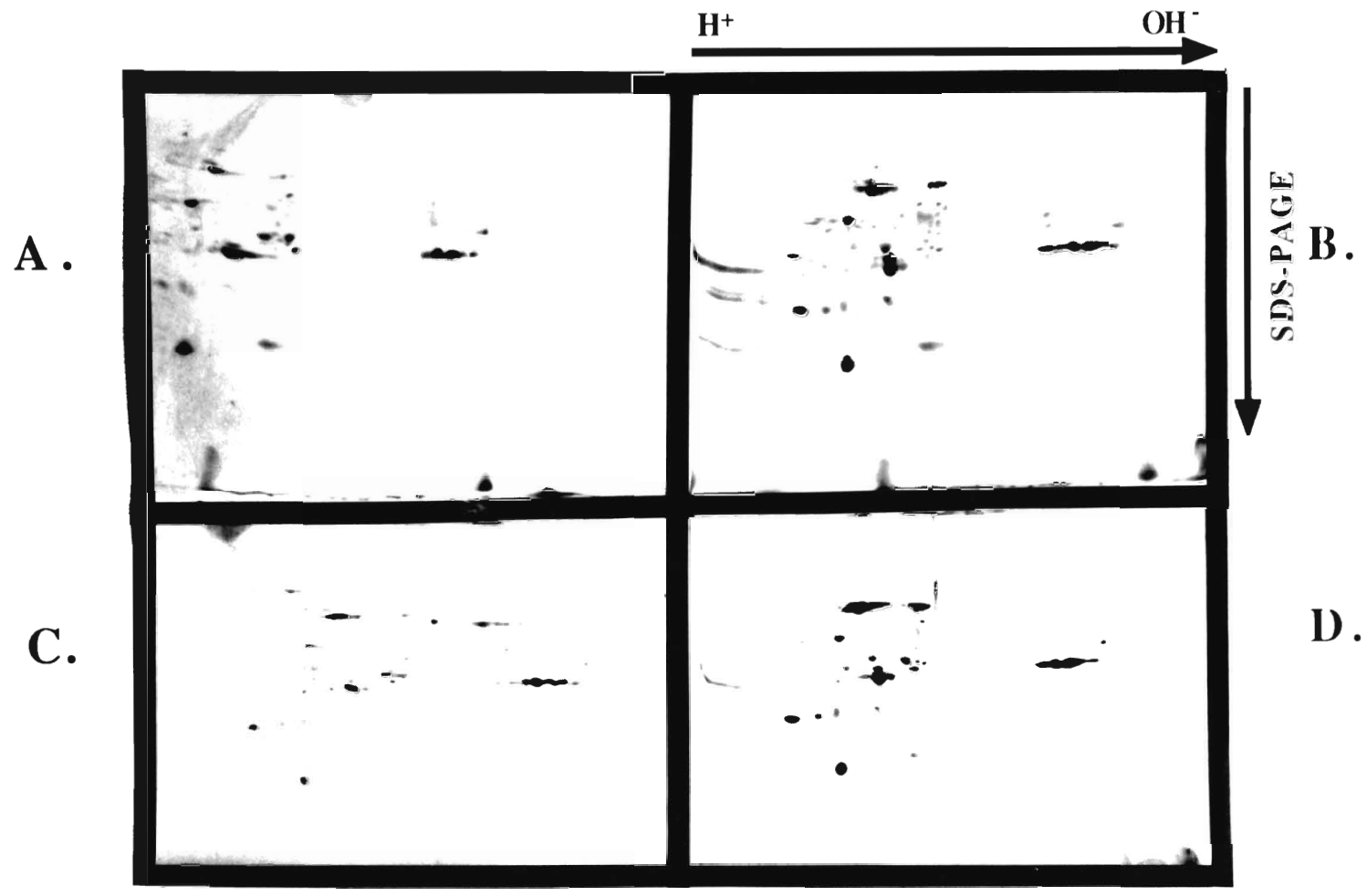


Figure XXI. Ethidium bromide stained DNA gels prior to transfer and Southern hybridization. (A) represents DNA isolated from various organisms and digested with the restriction enzyme Eco R1. (B) represents DNA extracted from the newt and digested with the various restriction enzymes indicated. Lambda Hind3 digested DNA molecular weight markers (Kb) are indicated.

Figure XXI. Ethidium Bromide Stained Genomic Gels

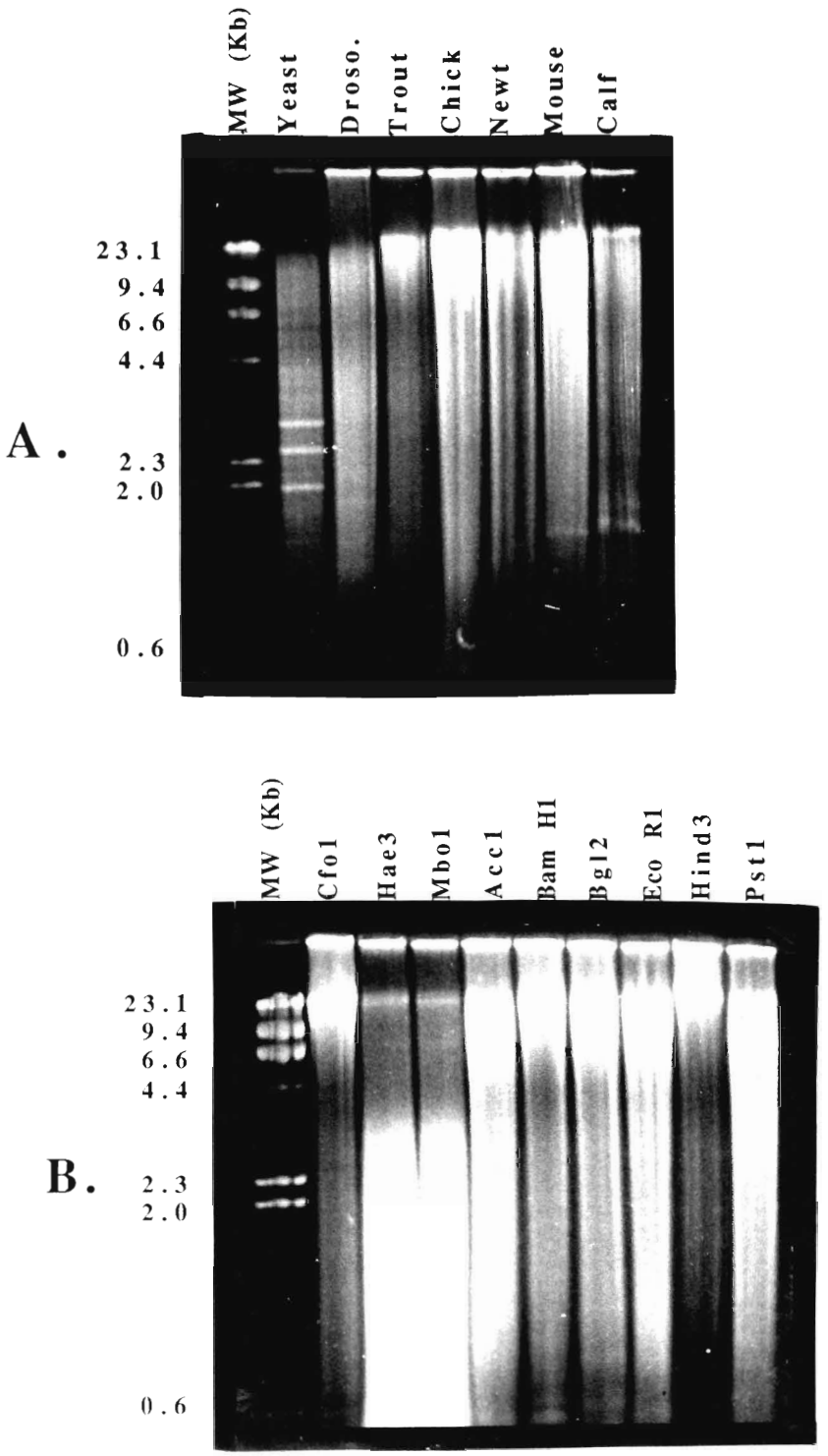


Figure XXII. Western immunoblot of protein (20µg) separated on a 10% SDS-polyacrylamide gel preceding its transfer to nitrocellulose. (A) represents the effects of a one hour heat shock and bilateral forelimb amputation through the humerus on hsp70 accumulation. The Western blot was probed with the monoclonal antibody (C92) which recognizes the inducible form of human hsp70. (B) control immunoblot to ascertain the specificity of the secondary conjugated antibody (GAM-AP) for the hsp70 antigen in the absence of the primary antibody (C92). Note the lack of hybridization in the heat shock and regenerate stages. (C) Amido black stained nitrocellulose showing high effeciency of protein transfer by electroblotting. The molecular weight markers are expressed in kDa. Arrows indicate the approximate position of the 70kDa proteins.

Cont.= unamputated control forelimb
HS= heat shock (34.5^{0}C)
1 h T= 1 h tail (p.f.a.)
1 h L= 1 h distal limb stump (p.f.a.)
6 h T= 6 h tail (p.f.a.)
6 h L= 6 h distal limb stump (p.f.a.)
1 d T= 1 day tail (p.f.a.)
1 d L= 1 day distal limb stump (p.f.a.)

Figure XXII. Western Hybridization Blot (C92)

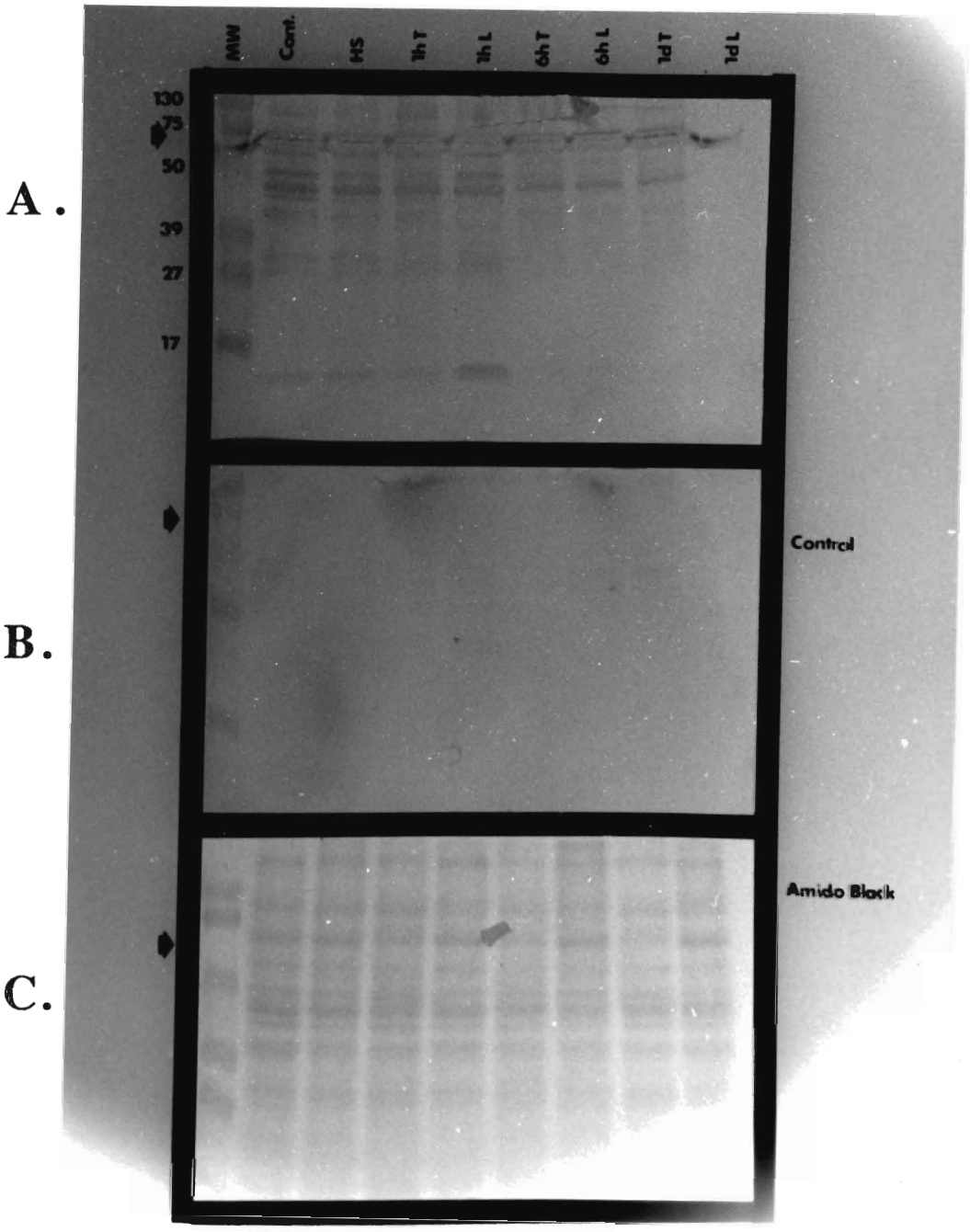
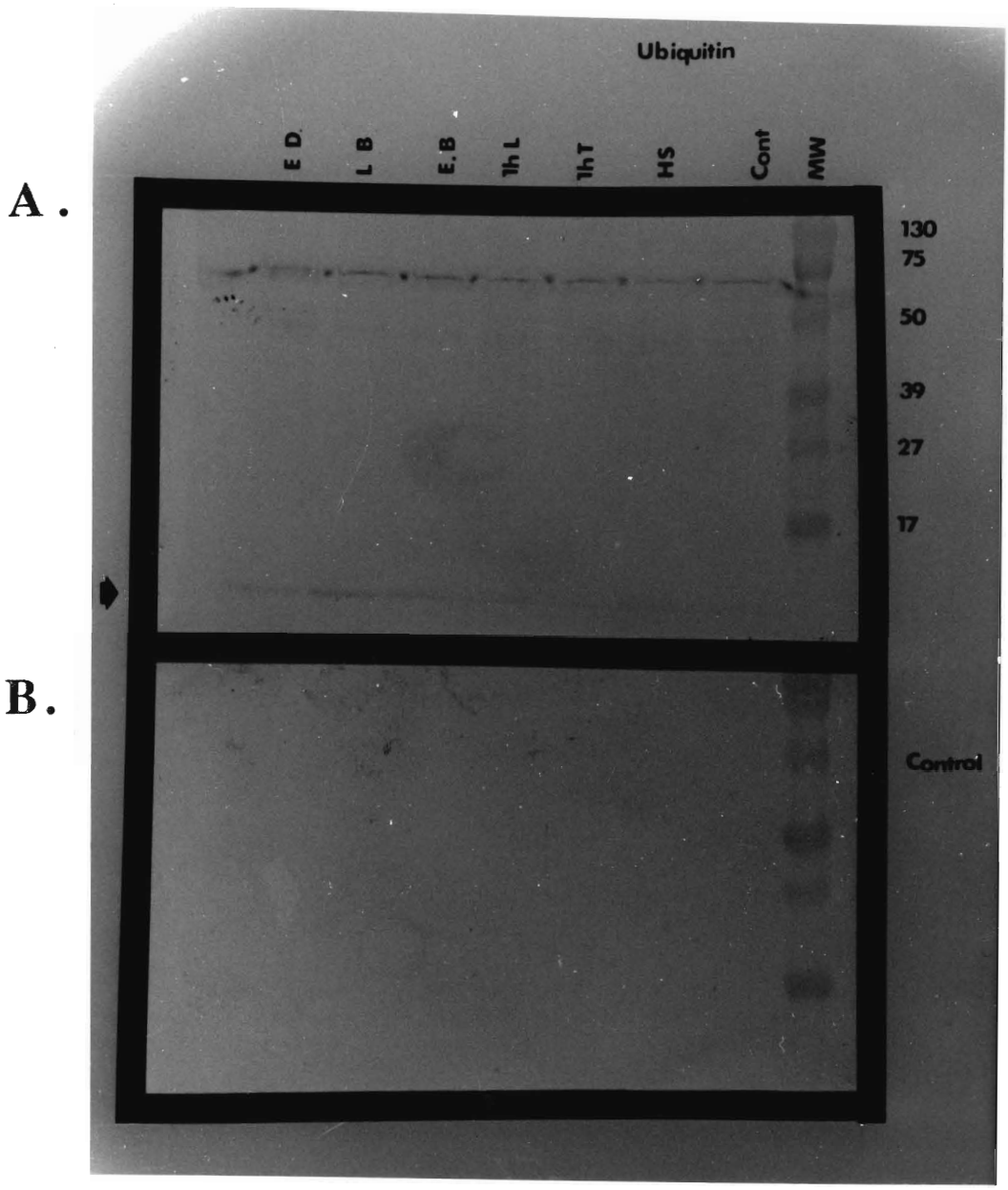


Figure XXIII. Western immunoblot of 15% SDS-PAGE probed with the monoclonal antibody (Ub1) which recognizes the human ubiquitin antigen. (A) represents the effects of a one hour heat shock and stage-specific levels of ubiquitin in the regenerating forelimb of the newt. (B) control immunoblot to ascertain the specificity of the secondary conjugated antibody (GAM-AP) for the ubiquitin antigen in the absence of the primary antibody (Ub1). The molecular weight markers are expressed in kDa.

Cont.= unamputated control forelimb
HS= heat shock (34.5^{0}C)
EB= early bud
LB= late bud
ED= early digits

The arrow in (A) points to the low levels of the 8.0kDa ubiquitin protein in the stage-dependent regenerate. Note, 20µg of protein was loaded per lane.

Figure XXIII. Ubiquitin Probed Western Blot



Enzymes, Solution and Buffers

L broth

10g bactotryptone
5g yeast extract
10g NaCl

Adjust volume to 1 litre with ddH_2O, autoclave.

LB plates

x vol. L broth
1.4% agar

Autoclave, pour when cooled to approximately 50^0C.

10% SDS (Sodium dodecyl sulfate)

100g electrophorsis-grade SDS
900ml sterilized ddH_2O, heat to assist dissolution
pH to 7.2 with conc. HCl

Adjust volume to 1 litre with autoclaved ddH_2O.

5M potassium acetate, pH4.8

60ml 5M potassium acetate
11.5ml glacial acetic acid
28.5ml ddH_2O

Autoclave, resulting solution is 3M with respect to potassium and 5M with respect to acetate.

1xTE, pH7.2 (Tris-EDTA)

10mM Tris-Cl, pH7.0
1mM EDTA, pH8.0

Adjust volume with ddH_2O, autoclave.

10mg/ml DNase free RNase

Dissolve pancreatic RNase A (Boehringer Mannheim) of concentration 10mg/ml in 10mM Tris-HCl, pH7.5 and 15mM NaCl. Adjust volume with sterile DEPC.ddH_2O, heat to 100^0C for 15 min, cool to room temperature, store at -20^0C.

STE

10mM Tris-Cl, pH7.6
100mM NaCl
1mM EDTA, pH8.0

Adjust volume with ddH_2O, autoclave.

0.1% DEPC.ddH_2O (Diethyl pyrocarbonate)

1ml DEPC into 1 litre of

Dissolve into solution by stir bar rotation, autoclave.

10mg/ml Proteinase K

dissolve Proteinase K (Boehringer Mannheim) of concentration 10mg/ml in:

5% SDS
200mM EDTA, pH8.0
100mM Tris-Cl, pH7.5

Adjust volume with autoclaved ddH_20, store at -70^{0}C.

Proteinase K buffer

dissolve:
50mM Tris-HCl, pH8
100mM EDTA, pH8
100mM NaCl
1% SDS

Filter sterilize and store aliquots at -20^{0}C.

Penol:chloroform:isoamyl alcohol (25:24:1)

To 1xTE saturated phenol (ultra-pure) of neutralized pH, appropriate volumes of ddH_20 saturated chloroform (analytical) and isoamyl alcohol were added. P:C:IA was stored in the dark at 4^{0}C.

chloroform:isoamyl alcohol (24:1)

To 24 volumes of ddH_20 saturated chloroform, one volume of isoamyl alcohol was added. Stored at room temperature in the fume hood.

10xNTB (nick translation buffer)

0.5M Tris-Cl, pH7.2
100mM $MgSO_4$
1mM dithiothreitol
500µg/ml BSA (bovine albumin)

Adjust volume with ddH_20, filter through a 0.45µm pore size Millipore filter, store at -20^{0}C.

1mg/ml DNase I

dissolve DNase I (1mg/ml) in:

150mM NaCl
50% sterile glycerol

Adjust volume with ddH_20, store at -20^{0}C.

20xSSC

175.3g NaCl
88.2g Na tricitrate
pH to 8.0 with NaOH

Adjust volume to 1 litre with ddH_20, autoclave.

Denatured, herring sperm DNA

Dissolve the DNA (concentration 10mg/ml) in sterile ddH_2O. Shear the DNA by passing it through an 18-gauge hypodermic needle several times. Boil for 10 min and store at -20^0.

1M Tris-Cl, pH7.6

121.1g Tris base in 800ml ddH_2O
pH to 7.6 with conc. HCl
Adjust volume to 1 litre with ddH_2O, autoclave.

100mM Sodium Phosphate buffer, pH6.8.

mix:
51ml 0.2M NaH_2PO_4, pH4.2
49ml 0.2M Na_2HPO_4, pH9.2
Adjust volume to 2 litres with DEPC.ddH_2O. A ten to one dilution of this will produce a 10mM sodium phosphate buffer, pH6.8.

50% Sodium Dextran

25g sodium dextran (ultra-pure) in 25ml sterile ddH_2O
Dissolve over low heat with constant agitation. Adjust volume to 50ml and aliquot into 5ml samples.

10xTBE

108g Tris base
55g boric acid
40ml 0.5M EDTA, pH8.0
Adjust volume to 1 litre and autoclave. Electrophoresis is conducted using 1xTBE.

10mg/ml Ethidium bromide

Add the desired volume of ddH_2O to achieve a ethidium bromide concentration of 10mg/ml. Store in a light resistant container and use at a final concentration of 0.5µg/ml.

Gel loading dyes

dissolve in sterile ddH_2O (w/v):
0.25% bromophenol blue
0.25% xylene cyanol
15% ficol (type 400)
Sterilize through a 0.45µm Millipore filter.

0.5EDTA, pH8.0

to 600ml ddH_2O dissolve:
168.1g EDTA
20g NaOH
Adjust pH to 8.0 with 10N NaOH and dilute to 1 litre prior to autoclaving.

10N NaOH

Dissolve 40g NaOH into ddH_20 and adjust to 100ml.

Sephadex G-50

Slowly add 30g sephadex G-50 (medium) to 250ml of 1xTE, pH8. Autoclave and decant cooled supernatant, replacing it with an equal volume of 1xTE, pH8. Store at 4^0C with 1ml of chloroform.

Deionization

To deionize glyoxal (6M) or formamide (neat), place 50ml of solution into a beaker containing 5g mixed-bed ion-exchange resin (e.g. BioRad, AG 501-X8,20-50). Cover the mixture and stir for 30 min. Add fresh activated resin to the filtrate. Aliquot the deionized filtrate and store frozen at -70^0C.

30% Acrylamide/Bis (2.67%C)

29.2g acrylamide (electrophoresis-grade)
0.8g N'N'-Bis-methylene-acylamide

Make to 100ml with sterile ddH_20. Filter (0.45µm) and store at 4^0C in the dark.

Sample buffer (SDS reducing buffer)

add to 4.0ml ddH_20:
1.0ml 0.5M Tris-HCl, ph6.8
0.80ml glycerol (ultra-pure)
1.6ml 10% SDS
0.4ml 2-ß mercaptoethanol
0.05% (w/v) bromophenol blue

Dilute the protein sample at least 1:4 with sample buffer, and heat at 95% for 4 min. Sample buffer half-life is approximately two months.

5x Electrode (SDS-PAGE running) buffer, pH8.3

9.0g Tris-base
43.2g glycine
3.0g SDS

Adjust volume to 600ml and store at 4^0C. Dilute 5x stock to 1x for electrophoresis.

Fixative and destain (SDS-PAGE)

400ml MeOH
100ml HOAc

Adjust volume to 1 litre.

0.1% Coomassie Blue (w/v)

Dissolve 0.5g Coomassie blue R-250 into 500ml of fixative. Filter through 1MM Whatman (twice). Stain for approximately 30 min.

Transfer buffer (Western blot)

12.08g Tris-base (ultra-pure)
56.7g glycine (electrophoresis grade)
800ml glass-distilled MeOH

Adjust to 4 litres with ddH_2O and pre-chill to 4^0C prior to use.

Amido black stain

0.1% amido black (w/v)
10% HOAc (v/v)
45% MeOH (v/v)

Adjust with ddH_2O.

Destain (Amido black stain)

2% HOAc (v/v)
90% MeOH (v/v)

Adjust with ddH_2O.

Tris buffered saline (1xTBS)

dissolve in ddH_2O:
4.84g Tris-base (ultra-pure)
58.48g NaCl

Adjust volume to 2 litre and the pH to 7.5 with conc. HCl.

Tween-20/TBS (1xTTBS)

Add 0.5ml Tween-20 to 1 litre TBS.

Blocking solution

Add 3g gelatin to 100ml TBS. Warm to 37^0C to dissolve gelatin, then cool.

Antibody buffer

Add 2g gelatin to 200ml TTBS. Warm to 37^0C to dissolve gelatin, then cool. Used in the dilution of the primary and secondary antibodies.

Carbonate buffer

dissolve in 900ml ddH_2O:
8.4g $NaHCO_3$
0.203g $MgCl_2.6H_2O$

Adjust to pH9.8 with 10N NaOH and dilute to a final volume of 1 litre.

BCIP/NBT

To vial A, add 30mg NBT to a 1ml solution of 70% DMF (N,N-dimethylformamide). In a second vial dissolve 15mg of BCIP in 1ml DMF and label it solution B. Just prior to colour development, mix solution A and B together and dilute to 100ml with ddH_2O.

SDS/ßME

mix:
0.3ml 10% SDS
0.1ml 2-ß mercaptoethanol
0.6ml ddH_2O

Store in 0.1ml aliquots at -70^{0}C.

2DSß

mix together:
5.97g urea (Boehringer Mannheim)
4ml 10% NP-40 (deionized)
0.40ml 40% ampholytes pH 3.5-9.5 (LKB)
0.10ml 40% ampholytes pH 6-8 (LKB)
0.154g dithiothreitol
1.12ml ddH_2O
add a trace of phenol red

Dissolve briefly at 37^{0}C and filter through 0.45µm Millipore. Dispense into 1ml aliquots and store at -70^{0}C.

pI Gel solution

2.75g urea
2.0ml 10% NP-40
0.5ml 30% acrylamide
0.675ml 1% bisacrylamide
0.20ml of 40% ampholytes pH3.5-9.5
0.05ml of 40% ampholytes pH6-8

Filter, degass into 1.2ml aliquots and store at -70^{0}C. There is no addition of TEMED and only 10% ammonium persulphate (8µl/1.2ml pI gel solution) is required for polymerization.

Overlay

mix together:
0.9ml 2SDß
0.05ml 2-ß mercaptoethanol
0.05ml ddH_2O

Make fresh daily.